	Type	L #	Hits	Search Text	DBs	Time Stamp	Comments
1	IS&R	L1	20338	(435/4-7.92).CCLS.	USPAT	2003/05/31 17:00	
2	IS&R	L2	860	(435/287.1).CCLS.	USPAT	2003/05/31 17:01	
3	IS&R	L3	4925	(436/518-532).CCLS.	USPAT	2003/05/31 17:01	
4	IS&R	L4	1120	(422/68.1).CCLS.	USPAT	2003/05/31 17:01	
5	BRS	L5	23079	1 or 2 or 3 or 4	USPAT	2003/05/31 17:01	
6	BRS	L6	1052	5 and (DNA same microtiter)	USPAT	2003/05/31 17:01	
7	BRS	L7	358	5 and (DNA with microtiter)	USPAT	2003/05/31 17:01	
8	BRS	L8	294	5 and ((DNA with microtiter) and antibody)	USPAT	2003/05/31 17:02	
9	BRS	L9	231	5 and ((DNA with microtiter) and antibody and (screen or screening))	USPAT	2003/05/31 17:02	
10	BRS	L10	24	5 and ((DNA with microtiter) same (screen or screening) and antibody)	USPAT	2003/05/31 17:04	
11	BRS	L11	27	5 and ((DNA with microtiter) same (screen or screening))	USPAT	2003/05/31 17:05	
12	BRS	L12	3	11 not 10	USPAT	2003/05/31 17:04	
13	BRS	L14	24	5 and ((DNA with microtiter) same (immobilize or immobilizedor immobilization or crosslink or crosslinking))	USPAT	2003/05/31 17:05	
14	BRS	L15	71	5 and ((DNA with microtiter) same (immobilize or immobilized or immobilization or crosslink or crosslinking))	USPAT	2003/05/31 17:05	
15	BRS	L13	24	5 and ((DNA with microtiter) same (immobilize or immobilization or crosslink or crosslinking))	USPAT	2003/05/31 17:08	

	Type	L #	Hits	Search Text	DBs	Time Stamp	Comments
16	BRS	L16	47	15 not 13	USPAT	2003/05/31 17:08	

	Type	L #	Hits	Search Text	DBs	Time Stamp	Comments
1	IS&R	L1	4	(("5849493") or ("5958696") or ("5705344") or ("5563036")).PN.	USPAT	2003/05/31 16:01	
2	BRS	L2	3	1 and microtiter	USPAT	2003/05/31 16:08	
3	BRS	L3	4	1 and dna	USPAT	2003/05/31 16:05	
4	BRS	L4	3	1 and antibody	USPAT	2003/05/31 16:03	
5	BRS	L5	47	dna-pk	USPAT	2003/05/31 16:06	
6	BRS	L6	13	5 and microtiter	USPAT	2003/05/31 16:11	
7	BRS	L7	19	dna same (uv or ultraviolet or damage or damaged or nicked) same (microtiter)	US-PGPUB; EPO; JPO; DERWENT	2003/05/31 16:20	
8	BRS	L8	49	dna same (uv or ultraviolet or damage or damaged or nicked) same (microtiter)	USPAT	2003/05/31 16:14	
9	BRS	L9	48	dna-pk	US-PGPUB; EPO; JPO; DERWENT	2003/05/31 16:21	
10	BRS	L10	13	dna-pk	EPO; JPO; DERWENT	2003/05/31 16:21	

US005958696A

United States Patent [19]

Crute

[11] **Patent Number: 5,958,696**

[45] **Date of Patent: Sep. 28, 1999**

[54] **QUANTITATIVE SOLID PHASE HELICASE ASSAY**

[75] Inventor: **James J. Crute**, Danbury, Conn.

[73] Assignee: **Boehringer Ingelheim Pharmaceuticals, Inc.**, Ridgefield, Conn.

[21] Appl. No.: **09/083,478**

[22] Filed: **May 22, 1998**

Related U.S. Application Data

[60] Provisional application No. 60/052,809, Jul. 17, 1997.

[51] **Int. Cl.**[6] **C12Q 1/68**; C12N 9/00; G01N 33/566

[52] **U.S. Cl.** **435/6**; 435/183; 436/501

[58] **Field of Search** 435/6, 183; 436/501

[56] **References Cited**

U.S. PATENT DOCUMENTS

5,705,344 1/1998 Giordano et al. 435/6
5,712,383 1/1998 Sheridan et al. 536/24.3

OTHER PUBLICATIONS

Kolalowski et al. "Comparison of an Intercalating Dye and an Intercalant–Enzyme Conjugate for DNA Detection in a Microtiter–Based Assay", Anal. Chem, vol. 68, pp. 1197–1200 Apr. 1, 1996.

Sambrook et al. Molecular Cloning:A laboratory manual, 2nd 2d. Cold Spring Harbor University Press, p. 4.9, 1989.

Abdel–Monem M., et al. Eur.J.Biochem. (1976) 441–449, 65.

Ventkatesan, M., et al. J. Biol.Chem. (1982) 12426–12434, 257.

Matson, S.W., et al. J.Biol.Chem. (1983) 14017–14024, 258.

Houston,P., and Kodadek, T. Proc.Natl.Acad.Sci.USA (1994) 5471–5474, 91.

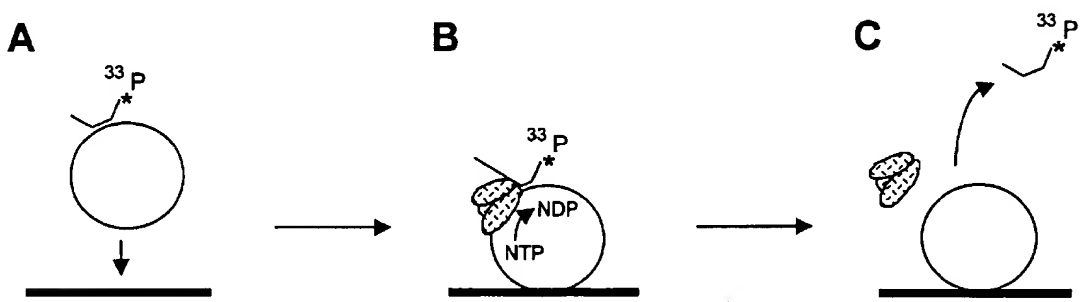
Raney,K.D., et al. Proc.Natl.Acad.USA (1994) 6644–6648, 91.

Primary Examiner—Lisa B. Arthur
Assistant Examiner—Jehanne Souaya
Attorney, Agent, or Firm—Robert P. Raymond; Anthony P. Bottino; Alan R. Stempel

[57] **ABSTRACT**

This invention relates to a solid phase helicase assay for identifying helicase inhibitors. The assay having a model helicase substrate adsorbed on a solid support, the model helicase substrate being an immobilized extended single-stranded nucleic acid polymer hybridized to a labeled helicase reaction product. The presence of the labeled helicase reaction product is detectable in solution on helicase activity. Also described is a method for measuring the helicase inhibiting ability of test substances thus, making the assay useful for identifying pharmaceutically important helicase inhibitors.

10 Claims, 6 Drawing Sheets



102(e)
1-3, 5, 12-15, 17

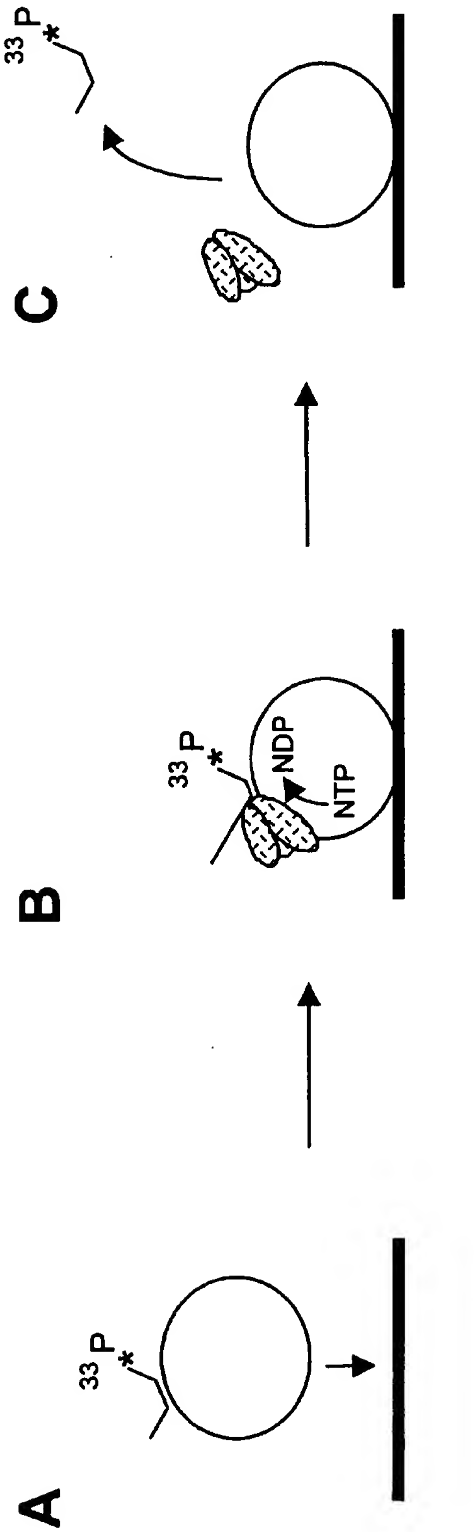


Fig. 1

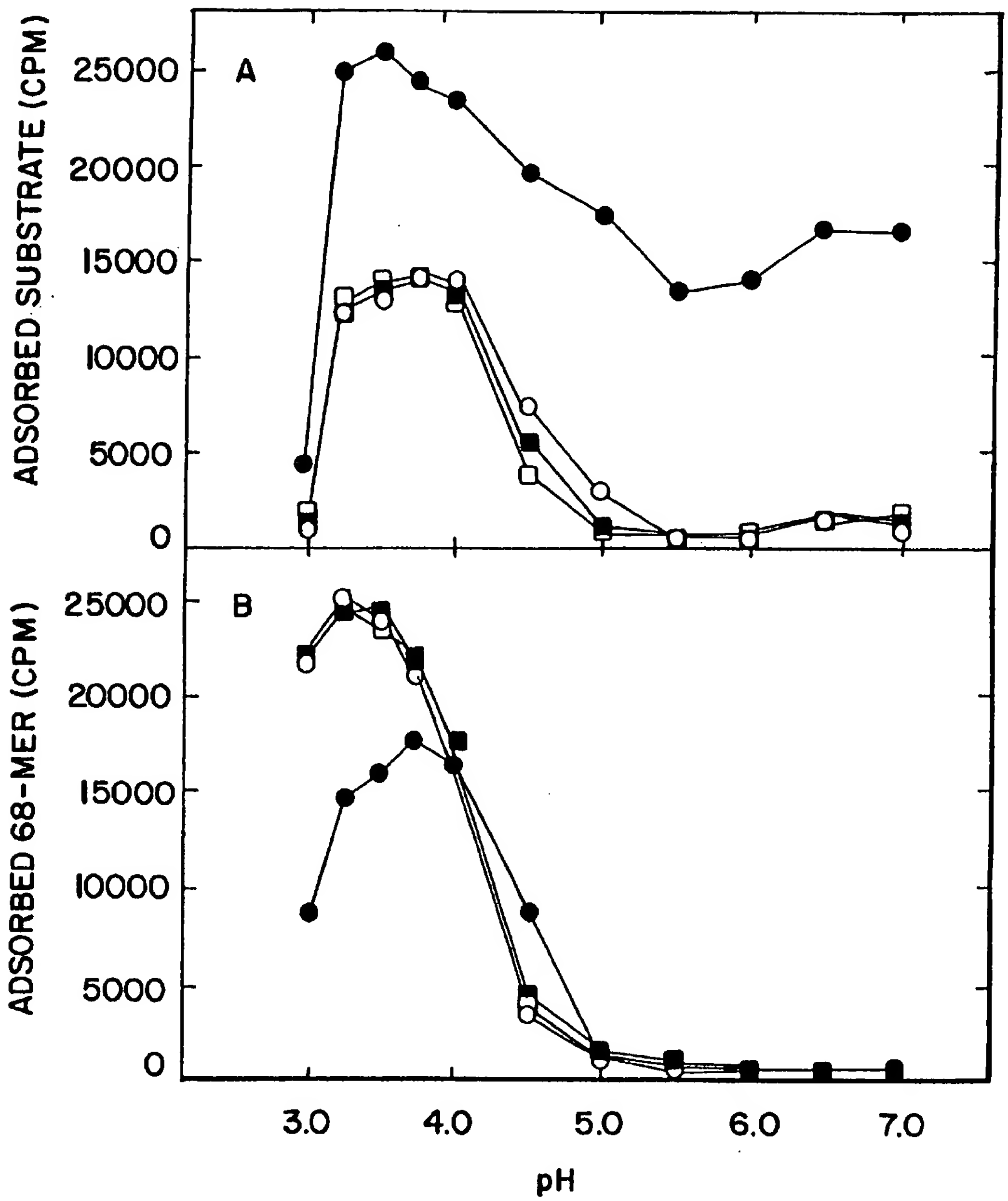

ADSORBED SUBSTRATE (CPM)
25000
20000
15000
10000
5000
0
A
ADSORBED 68-MER (CPM)
25000
20000
15000
10000
5000
0
B
3.0
4.0
5.0
6.0
7.0
pH

Fig. 2

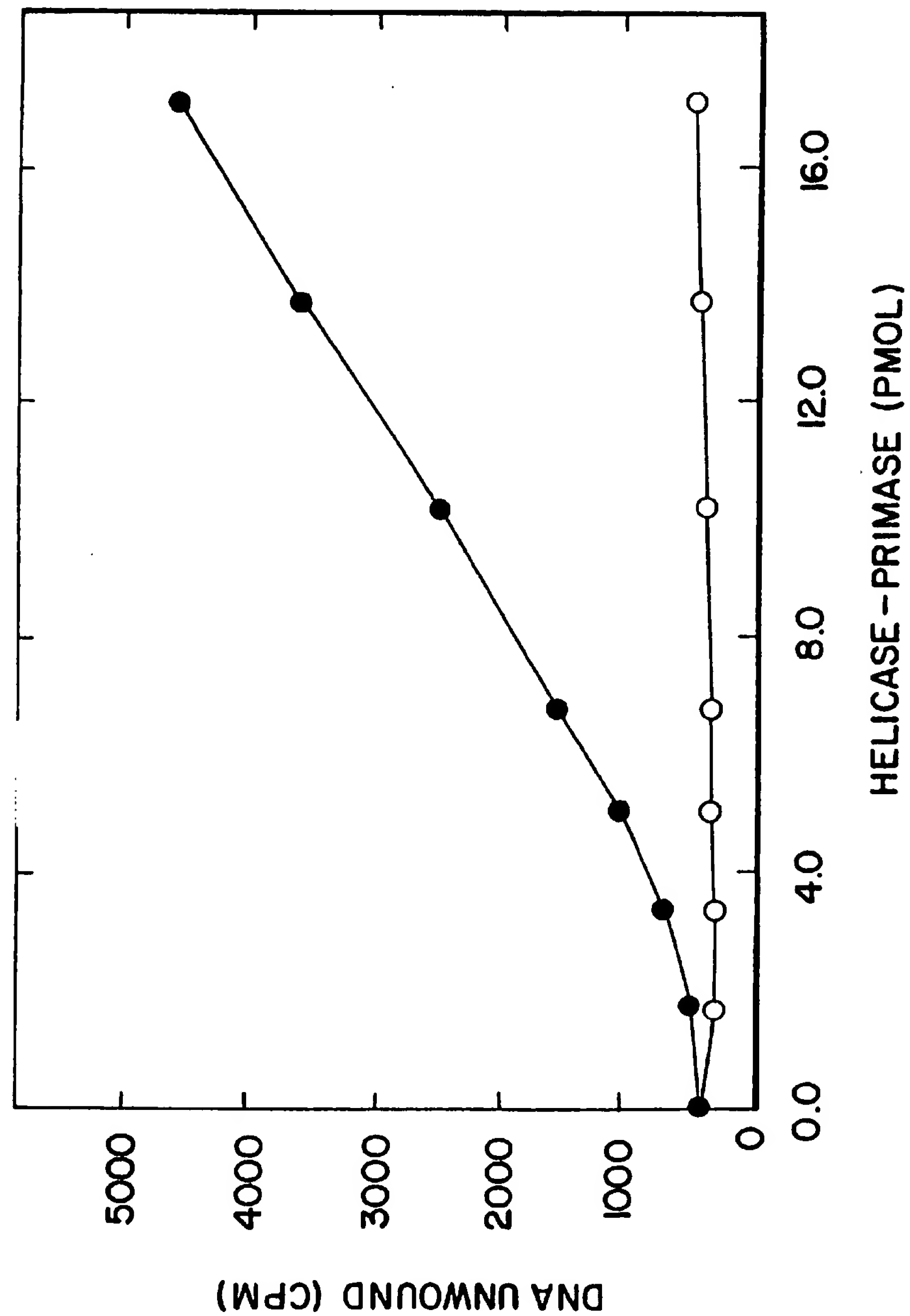

DNA UNWOUND (CPM)
0
1000
2000
3000
4000
5000
HELICASE-PRIMASE (PMOL)
0.0
4.0
8.0
12.0
16.0

Fig. 3

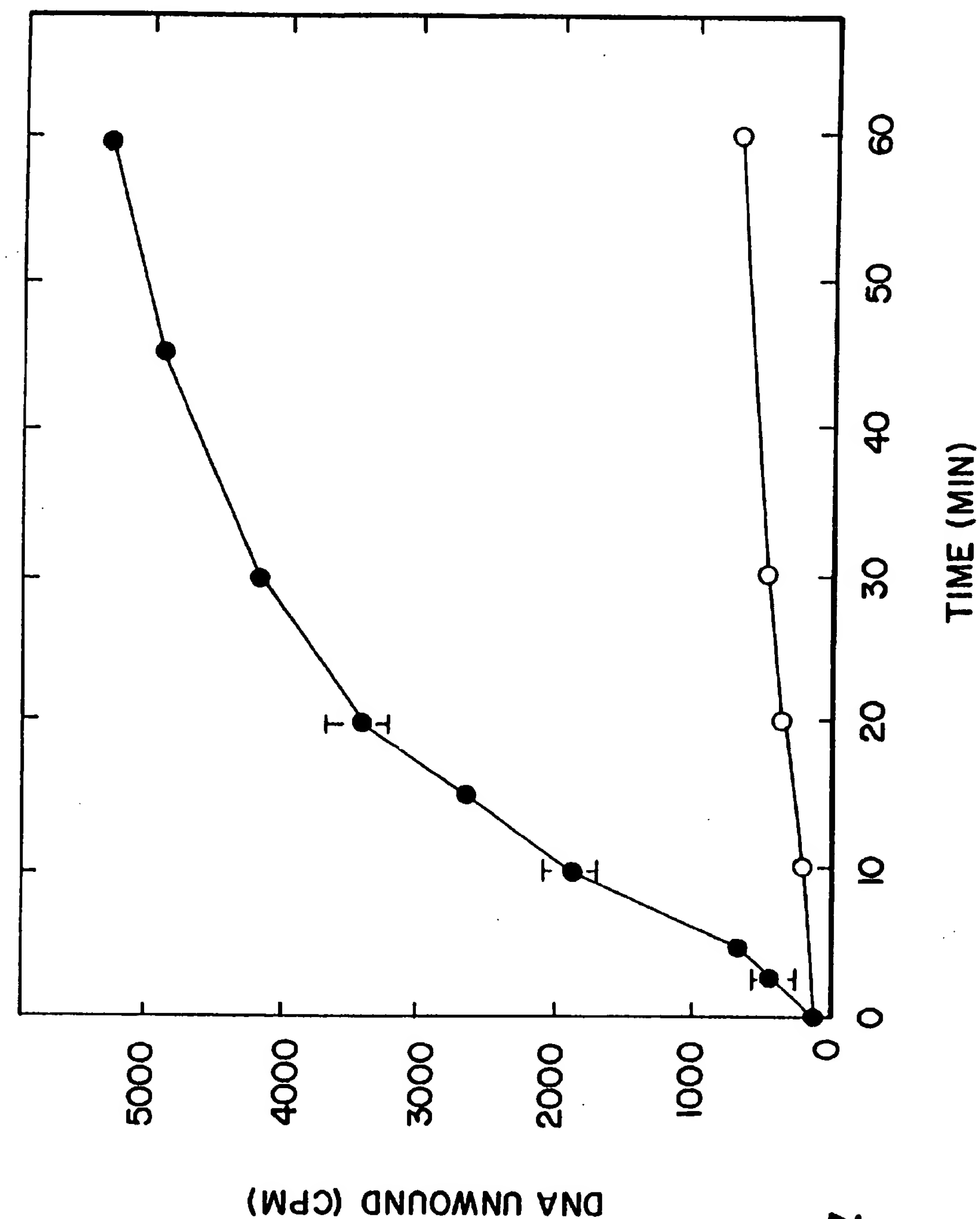

DNA UNWOUND (CPM)
0
1000
2000
3000
4000
5000
TIME (MIN)
0
10
20
30
40
50
60

Fig. 4

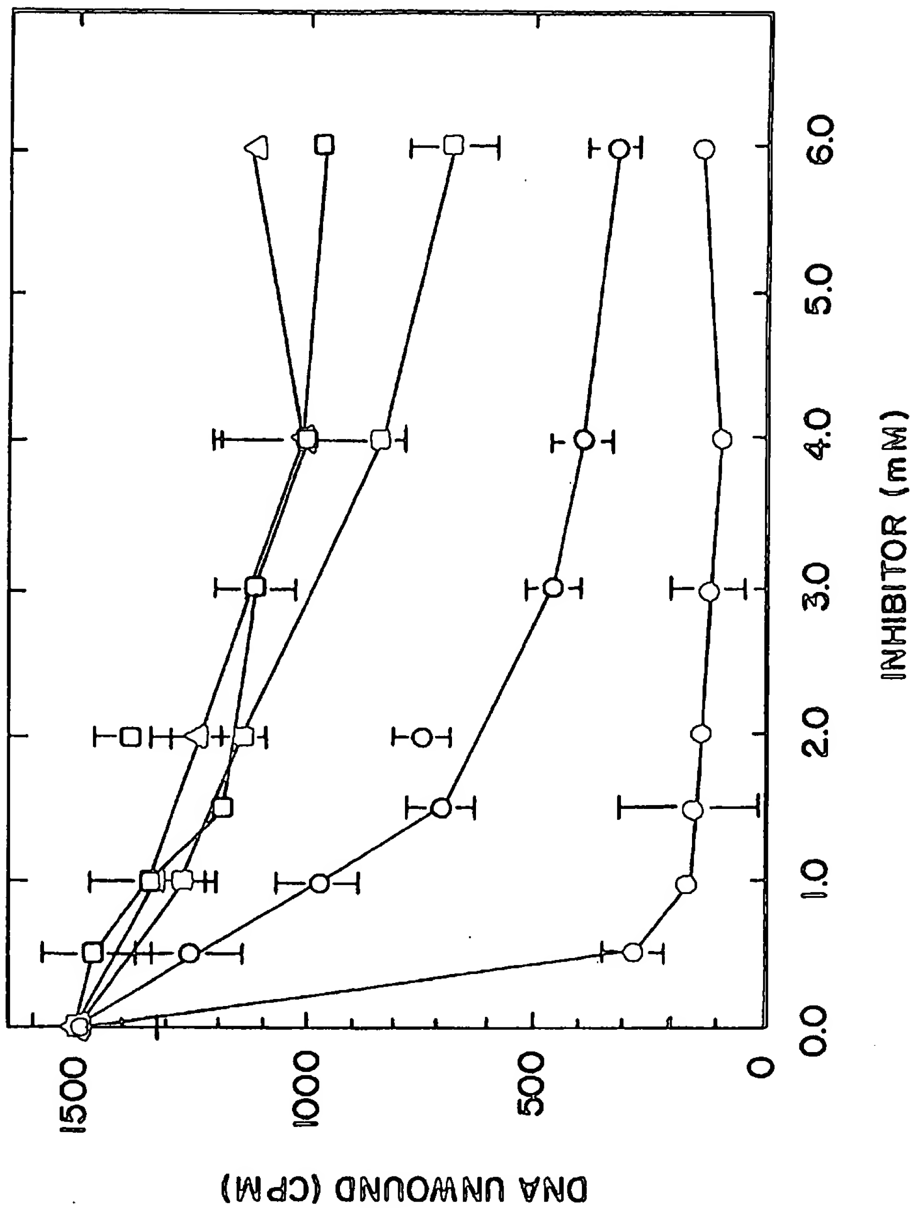

DNA UNWOUND (CPM)
1500
1000
500
0
INHIBITOR (mM)
0.0
1.0
2.0
3.0
4.0
5.0
6.0

Fig. 5

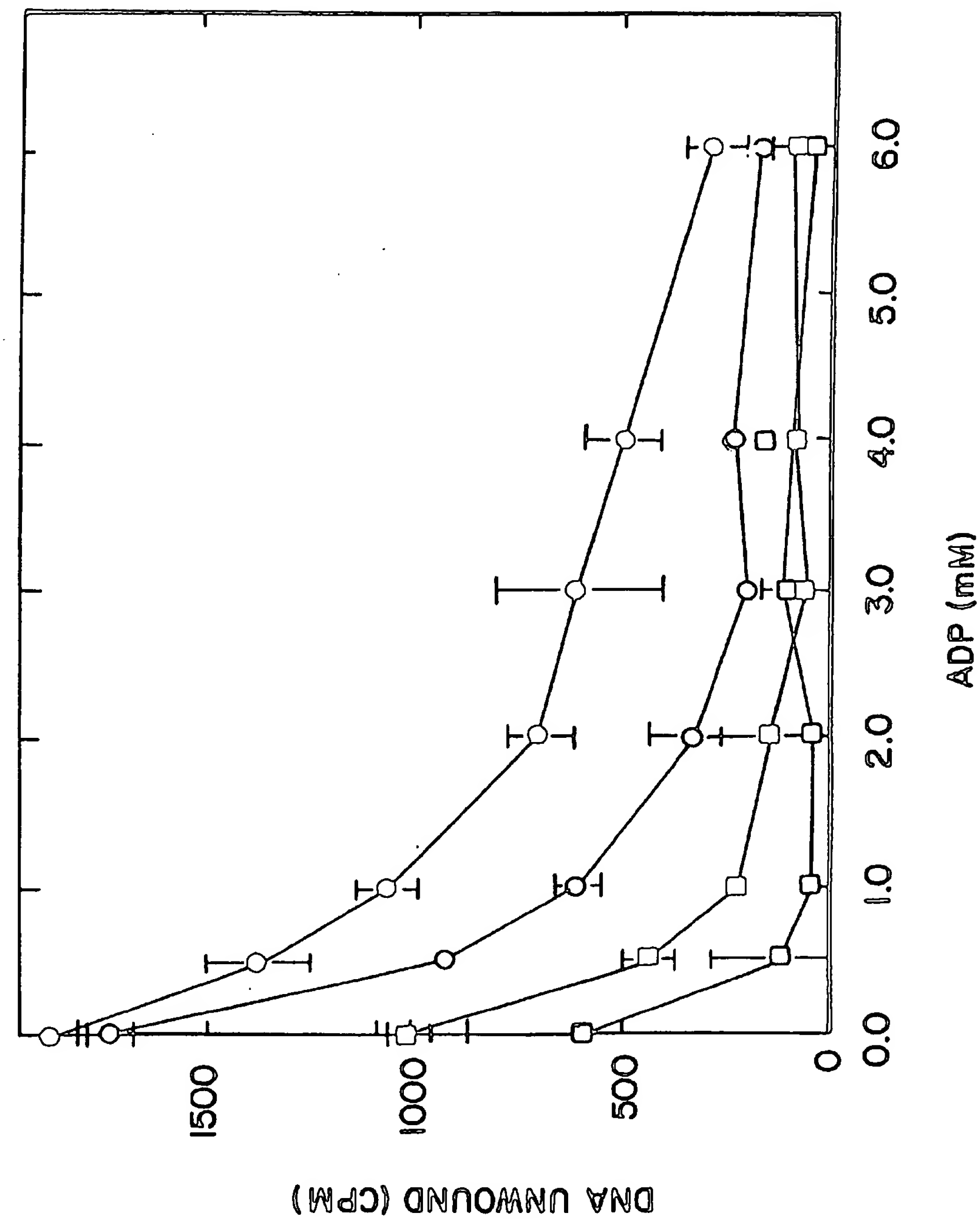


Fig. 6

QUANTITATIVE SOLID PHASE HELICASE ASSAY

This application claims the benefit of U.S. Provisional Application No. 60/052,809 filed Jul. 17, 1997.

TECHNICAL FIELD OF THE INVENTION

This invention relates to a solid phase assay to measure quantitatively the ability of a test compound to inhibit DNA or RNA helicase activity. This assay advantageously simplifies the quantitation of helicase activity, and is especially well suited for rapid quantitation of large numbers of individual samples. The assays and methods of this invention may be used to identify pharmaceutically important helicase inhibitors.

BACKGROUND OF THE INVENTION

Helicases are mechanochemical enzymes that couple the energy of nucleoside triphosphate hydrolysis to the dehybridization or unwinding of duplex nucleic acid molecules (1). Nucleic acid unwinding is of central importance in a variety of nucleic acid transactions that include the transcription, translation, recombination, and replication of genetic material. The importance of helicases is further underscored by the large number of DNA or RNA helicases identified in prokaryotic and eukaryotic organisms.

Assays that are both rapid and quantitative for the routine measurement of helicase activity have not been available. There have been essentially two types of helicase assays developed to date. One type of assay has relied on the generation of single-stranded, nuclease sensitive molecules from nuclease insensitive, uniformly radiolabeled double-stranded substrates, followed by the quantitation of acid soluble nucleotides (2). Alternatively, helicase activity has been determined by the unwinding of model duplex substrates comprised of a labeled oligonucleotide hybridized to a larger unlabeled single-stranded molecule followed by size separation (3, 4). Because of the difficulty in obtaining assay substrates suitable for performing the first type of assay, the latter assay has been more commonly utilized. The use of model duplex substrates has also generated the greater variety of nucleic acid structures necessary for the detection of activity associated with different classes of helicase enzymes.

Although the construction of model helicase substrates is useful for detecting enzyme-associated helicase activity, the measurement of that activity is unduly tedious when large numbers of assays need to be performed. This is due to the methods commonly employed in the quantitation of helicase reaction products. Helicase reaction products have primarily been separated from substrates by non-denaturing PAGE (4). Alternative methods have included the hybridization of single-stranded helicase-generated nucleic acid molecules to scintillant-containing beads or fluorescence methods based on resonance energy transfer properties of labeled nucleic acid molecules or specific double-stranded DNA dyes (5–8). In one recently published solution phase helicase assay, DNA intercalators were used as an indicator fluorescent dye for double-stranded DNA molecules (8). In that assay, helicase activity was measured by a decrease in fluorescence.

Each of the above-described quantitative methods suffers from individual drawbacks in terms of accuracy, reproducibility, use of special or sophisticated equipment, time required, expense and capacity to measure routinely large numbers of samples for helicase activity.

SUMMARY OF THE INVENTION

This invention overcomes the drawbacks associated with the previous helicase assays by providing a solid phase assay in which the product of the helicase reaction may be readily and quantitatively detected.

The prime objective of this invention is to provide a solid phase assay comprising a model helicase substrate adsorbed on a solid support, wherein the model helicase substrate comprises an immobilized extended single-stranded nucleic acid polymer hybridized to a labeled helicase reaction product, wherein the presence of the labeled helicase reaction product is detectable in solution on helicase activity.

Another objective of this invention is to provide a method for measuring the helicase inhibiting ability of a test substance comprising the steps of:

(a) immobilizing on a solid support a model helicase substrate comprising an extended single-stranded nucleic acid polymer hybridized to a labeled helicase reaction product, wherein the labeled helicase reaction product is detectable in solution on helicase activity;

(b) contacting the immobilized model helicase substrate with a test substance to produce a reaction premix;

(c) contacting the reaction premix of step (b) with a helicase capable of releasing the labeled helicase reaction product into solution on helicase activity; and

(d) measuring the amount of labeled helicase reaction product released into solution as a function of time.

A further objective of this invention is to provide inhibitors of helicase activity identified using the assays and methods of this invention.

These and other objectives will be readily appreciated by those of ordinary skill in the art based upon the following detailed disclosure of this invention.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1A, 1B, 1C. Schematic representation of the steps in the solid phase helicase assay. A. Adsorption of radiolabeled model helicase substrates to derivatized surfaces. B. Progression of the helicase reaction. C. Release of soluble, labeled helicase reaction products. Quantitation of the helicase reaction products by scintillation counting reaction mixture aliquots. NTP, nucleoside 5'-triphosphate; NDP, nucleoside 5'-diphosphate. The helicase enzyme is represented as a heterotrimer.

FIGS. 2A, 2B. Adsorption of model helicase substrates and helicase reaction products is pH and surface-dependent. Adsorption of nucleic acids and subsequent quantitation was carried out as described herein, wherein the pH of the citrate buffer used for adsorption was varied between 3.0 and 7.0 as indicated in FIG. 2. Surfaces used were derivatized with avidin (◉), streptavidin (○), glycine (□)or PBS (▣). Results are the mean of triplicate samples. Adsorption of helicase substrates (A), adsorption of the radiolabeled 68-mer helicase reaction product (B).

FIG. 3. Helicase enzyme and ATP are required for unwinding of adsorbed duplex substrates. Helicase substrate was adsorbed to avidin-derivatized 96 well plates for helicase reactions. Increasing amounts of the HSV-1 helicase-primase holoenzyme were added as indicated in either Buffer C (◉) or Buffer C with ATP deleted (○). Helicase activity was determined after 15 min of reaction time. Results are the mean±standard error of triplicate samples. Note: Error bars do not show beyond the points.

FIG. 4. Helicase activity is proportional to reaction time in the solid phase helicase assay. Helicase assays were

performed and results quantitated with avidin-derivatized plates. Helicase assays were assembled in Buffer C with 6.8 pmol of the HSV-1 helicase-primase holoenzyme per reaction (○) or in Buffer C without ATP (○) to determine background values. Results are the mean±standard error of triplicate samples.

FIG. 5. Helicase activity of the HSV-1 helicase-primase is prevented by non-specific enzyme inhibitors. Helicase assays were performed and results quantitated with avidin-derivatized plates. Assays were assembled in triplicate with 6.8 pmol HSV-1 helicase-primase holoenzyme per reaction in the presence of the indicated nucleoside derivative. Reactions were allowed to proceed for a period of 20 min. prior to termination and quantitation. Either ATPγS,(◉); ADP, (○); ADPβS, (■); AMPPNP, (□); AMPPCP, (Δ) was added at the indicated concentrations. A background value of 299 cpm (standard error=54 cpm, n=3) was subtracted from individual data points prior to the calculation of means and standard errors.

FIG. 6. Inhibition of the NTP-driven helicase activity of the HSV-1 helicase-primase by ADP. Helicase assays were performed and results quantitated with 6.8 pmol HSV-1 helicase-primase holoenzyme per reaction in the presence of the indicated nucleoside avidin-derivatized plates except that Buffer C contained 4.0 mM of one of the nucleoside triphosphates indicated below to drive the helicase reactions. ADP was added as a non-specific nucleoside helicase inhibitor at the indicated concentrations. A background value of 368 cpm (standard error=44 cpm, n=11) was subtracted from each point prior to the determination of means and standard errors. Helicase assays contained ATP (◉), GTP (○), CTP (▨), or UTP (□).

DETAILED DESCRIPTION OF THE INVENTION

In order that the invention herein described may be more fully understood, the following detailed description is set forth. As used herein, the following abbreviations apply:

ADPβS=adenosine-5'-O-(2-thiodiphosphate)
AMPPCP=adenylyl(β,γ-methylene)-diphosphonate
AMPPNP=adenylyl-imidodiphosphate
ATPγS=adenosine-5'-O-(3-thiotriphosphate)
BS^3=bis(sulfosuccinimidyl) suberate
BSA=bovine serum albumin
DTT=dithiothreitol
EGTA=ethylene glycol-bis(β-aminoethyl ether)-N,N'-tetraacetic acid
HSV-1=herpes simplex virus type 1
PAGE=polyacrylamide gel electrophoresis
PBS=phosphate-buffered saline
SDS=sodium dodecyl sulfate
SSB=single-stranded DNA binding protein.
Surfact-Amps 20=Tween 20

The solid phase assay of this invention comprises a model helicase substrate adsorbed on a solid support, wherein the model helicase substrate comprises an immobilized extended single-stranded nucleic acid polymer hybridized to a labeled helicase reaction product, wherein the presence of the labeled helicase reaction product is detectable in solution on helicase activity.

The assays of this invention are particularly well suited for identifying helicase inhibitors. Specifically, the assays of this invention are suitable for use in a method for measuring the helicase inhibiting ability of a test substance such as a test compound comprising the steps of:

(a) immobilizing on a solid support a model helicase substrate comprising an extended single-stranded nucleic acid polymer hybridized to a labeled helicase reaction product, wherein the labeled helicase reaction product is detectable in solution on helicase activity;
(b) contacting the immobilized model helicase substrate with a test substance to produce a reaction premix;
(c) contacting the reaction premix of step (b) with a helicase capable of releasing the labeled helicase reaction product into solution on helicase activity; and
(d) measuring the amount of labeled helicase reaction product released into solution as a function of time.

Solid support materials that can be used for this assay include any conventional support materials, including (but not limited to) polystyrene, polyvinyl chloride or polycarbonate microtiter plates or beads and derivatized argarose or acrylamide beads. The preferred support material for the assays of this invention is a polystyrene 96-well microtiter plate. The surface of the solid support material is preferably derivatized with a protein. However, other small DNA-interaction promoting materials (e.g., glycine) may also be used. In general, preferred derivatized surfaces for use in the assay of this invention are characterized by promoting (1) a stable interaction between the derivatized surface and the helicase substrate at acidic pH (i.e., between about 2 and about 5, but preferably between about 3 and about 4.5) and on transfer to neutral pH (i.e., between about 6 and about 8) (see FIG. 2A) and (2) a lack of binding affinity between the derivatized surface and the labeled helicase reaction product at neutral pH or slightly alkaline pH (i.e., between about 8 and about 10) (see FIG. 2B). Derivatized surfaces useful in the assays of this invention include those of cytochrome-C, avidin, streptavidin or glycine. The most preferred derivatized surface for use in the assays of this invention is an avidin-derivatized polystyrene microtiter plate.

The model helicase substrate for use in the assays and methods of this invention comprise two distinct components: (1) an extended single-stranded nucleic acid polymer which is hybridized to (2) a labeled helicase reaction product. The extended single-stranded nucleic acid polymer is further characterized as being adsorbable to the surface of a solid support material while retaining its ability to hybridize to the labeled helicase reaction product under suitable assay conditions. In general, the extended single-stranded nucleic acid polymer according to this invention is adsorbed to the solid support at acid pH and remains adsorbed on transition to neutral pH. Preferred extended single-stranded nucleic acid polymers are circular, single-stranded DNA chains from about 1000 to about 10,000 nucleotides in length (preferably, between about 3000 and about 10,000 nucleotides and more preferably, between about 5,000 and about 10,000 nucleotides in length).

In general, helicases (whatever the native organism) are not sequence specific and will unwind double-stranded nucleic acid polymers (DNA or RNA, depending on the nature of the helicase), regardless of the particular sequence presented. Accordingly, the sequence identity of the extended single-stranded nucleic acid polymer and likewise, the labeled helicase reaction product, may be freely varied, so long as the extended single-stranded nucleic acid polymer conforms to the above noted requirements and the labeled helicase reaction product remains hybridized to the extended single-stranded nucleic acid polymer at acidic pH and is released into and detectable in solution on helicase activity at neutral pH. Preferred model helicase substrates of this invention are optionally double-tailed. In double-tailed model helicase substrates, the labeled helicase reaction product comprises a hybridizing sequence flanked by non-hybridizing sequences on both its 5' and 3' end. Since

helicases are directional (i.e., some proceed in the 5'→3' direction while others proceed in the 3'→5' direction), such double tailed model helicase substrates may be more universally utilized because those model helicase substrates can facilitate helicase activity in either the 3' to 5' or the 5' to 3' direction. This type of substrate allows for a high degree of flexibility when performing assays since helicases that require free 3' or 5' fork-like structures may be interchangeably measured. Additionally, helicases not requiring fork-like structures to display activity may also be assayed using a double-tailed model helicase substrate. Preferably, the labeled helicase reaction product for use in the preferred double-tailed model helicase substrates of this invention will have from about 10–about 50 nucleotides (and preferably, from about 15–about 30 nucleotides) in the hybridizing region of its sequence and from about 10–about 80 (preferably, about 15–about 30) non-hybridizing nucleotides flanking both its 3' and its 5' end. FIG. 1A graphically represents such a double-tailed model helicase substrate being immobilized to a solid support.

The preferred model helicase substrate for use in the assays of this invention comprises M13mp19 single-stranded DNA hybridized with the ^{33}P-labeled 68-mer described in (9) and (10). The ^{33}P-radiolabeled 68-mer comprises 22 hybridizing nucleotides and 23 non-hybridizing nucleotides flanking the 3' and 5' ends of the hybridizing sequence. The use of a radiolabeled oligonucleotide annealed to M13mp19 single-stranded advantageously allows for the quantitation of unwound substrate by liquid scintillation, counting an aliquot of the assay solution from individual wells. Although this preferred helicase substrate may be used with any DNA helicase, it is especially well suited for measuring helicase activity of a herpes helicase-primase and, more preferably, the HSV-1 helicase-primase.

To assist in detection of the released helicase reaction product, the released helicase reaction product is preferably radiolabeled. Fluorescence and other conventional detection methods may also be used, so long as they are sufficiently sensitive and accurate to meet the needs of the investigator. Appropriate alternate detection methods are well known to those possessing ordinary skill in the art.

The assays and methods of this invention envision the use of RNA-, DNA- or mixed RNA/DNA model helicase substrates. For instance, the extended single-stranded nucleic acid polymer and labeled helicase reaction product may be made of either RNA or DNA. Furthermore, the extended single-stranded nucleic acid polymer may be made of RNA, while the labeled helicase reaction product may be made of DNA (or visa versa). Such DNA/DNA, DNA/RNA, RNA/DNA and RNA/RNA combinations will allow for the study of all types of helicases, including prokaryotic replicative helicases (such as the dnaB protein of *E. coli*) and various eukaryotic helicases. Although the examples described herein utilize the HSV-1 helicase-primase and a DNA/DNA model helicase substrate, it should be recognized that any helicase may be used in the assays and methods of this invention with a model helicase substrate appropriate for that helicase.

According to this invention, the extended single-stranded nucleic acid polymer component of the model helicase substrate is adsorbed to the surface of the solid support material at an acidic pH and that adsorption is not interrupted by a transition to neutral pH. Preferably, the labeled helicase reaction product is hybridized to the extended single-stranded nucleic acid polymer prior to immobilization. The labeled helicase reaction product component of the helicase substrate is released on helicase activity at neutral pH and remains detectable in solution. Advantageously, the released helicase reaction product is not adsorbed onto the support material nor does it rehybridize to the immobilized extended single-stranded nucleic acid polymer under conditions needed to induce helicase activity and to detect the released helicase reaction product. Helicase activity is, therefore, easily and rapidly quantitated by removing aliquots of the reaction solution over a period of time and detecting the presence of released helicase reaction product in the solution. Preferably, the aliquots are removed and analyzed in 30 second–10 minute increments over a 2 hour period. More preferably, the aliquots are removed and analyzed in 5–10 minute increments over a 1 hour period. As one possessing ordinary skill in this art will readily acknowledge, measurements may be made in longer or shorter time increments over a longer or shorter period of time, according to the particular objectives of a given experiment.

Without wishing to be bound by theory, the physical biochemical processes behind the adsorption of model helicase substrates to solid supports is likely to be at least partly hydrophobic in nature, since substrate adsorption is favored at moderate ionic strengths and not prevented at high ionic strengths. There may also be electrostatic interactions that participate in binding the nucleic acid substrates, as acidic pH is optimal for the binding of helicase substrates to protein-derivatized surfaces. The overall positive charge of the derivatized surface of the solid support tends to adsorb the overall negatively charged extended single-stranded nucleic acid polymers. However, even when the ionic interaction is altered (e.g., on addition of salt to the reaction premix), the extended single-stranded nucleic acid polymer does not disassociate from the surface, implicating possible non-ionic (e.g., hydrophobic) interactions.

Activity in the solid phase helicase assay of this invention depends on the addition of a nucleoside triphosphate, generally ATP, to drive the unwinding reaction. In addition, the rate of reaction is mediated by the amount of enzyme added to the reaction premix and the kinetic rate constant of the enzyme. For example, unwinding activity measured in the solid phase helicase assay using the HSV-1 helicase-primase is generally proportional between about 3 and at least 16 pmol of added HSV-1 enzyme and linear with time up to 30 minutes. However, it would be expected that with different rate constants, the rate of unwinding activity might be significantly different. The appropriate parameters (e.g., time intervals, amount of enzyme or test compound used, etc.) can be adjusted by those possessing ordinary skill in the art to arrive at a suitable assay system for a given purpose.

As the following examples demonstrate, the assays and methods of this invention have been used to identify nucleoside-based HSV-1 helicase-primase inhibitors from the commonly available nucleosides AMPPCP, AMPPNP, ADPβS, ADP, and ATPγS. AMPPCP and AMPPNP were found to be ineffective at inhibiting the helicase activity of the HSV-1 helicase-primase, ADPβS was a weak helicase inhibitor, and ADP and ATPγS effectively inhibited helicase activity to near background levels at concentrations of 0.5 mM and 6.0 mM, respectively. Inhibition of the HSV-1 helicase-primase by either ADPβS, ADP, or ATPγS was found to be concentration-dependent. The potency of these nucleoside helicase inhibitors was also analyzed in more traditional helicase assays where substrates were separated from products by non-denaturing PAGE. Results were similar to those found with the solid phase helicase assay, providing a good correlation and further validating the assay of this invention. These results support the hypothesis that

these nucleosides inhibit helicase action by competition with the NTP that drives helicase activity.

The inhibitory activity of ADP against the HSV-1 helicase-primase has also been characterized. ADP was a more potent and effective inhibitor when GTP, CTP or UTP was used to drive the helicase reaction when compared to reactions utilizing ATP. Interestingly, the relative effectiveness of each of the four nucleoside triphosphates in supporting helicase activity was identical to their relative abilities to be hydrolyzed by the HSV-1 helicase-primase in DNA-dependent NTPase assays. This observation is again consistent with a mechanism of inhibition wherein ADP competes with the NTP used to drive the helicase reaction.

The solid phase helicase assay of this invention advantageously measures both accurately and quickly the extent to which a test substance inhibits helicase activity. For example, once the microtiter plates used for the assays were avidin-derivatized and helicase substrates adsorbed, the experiments depicted in FIGS. **5** and **6** were assembled, run, and processed in a period of only 2 hours each. As these experiments contain nearly 200 individual helicase reactions, the improvement in efficiency is substantial compared to alternative methods. Further time efficiency could be introduced by using scintillation counters capable of analyzing 96 well plate formats. Helicase reaction solutions could be removed from the solid phase assay plates and directly dispensed to scintillant-containing plates for quantitation. Importantly, this increase in through-put of the solid phase helicase assay does not sacrifice accuracy. All of the assays reported below were performed in triplicate with standard errors less than 5% of the mean values. In fact, in many circumstances the standard errors were less than the size of the points used in data plotting (see FIGS. **3** and **4**).

Since the methods described for the solid phase helicase assay are readily carried out and do not require specialized equipment, the assay of this invention should be very useful in the preliminary characterization of new helicases. The experimental methods exemplified and described herein, with or without modifications to adapt to individual helicase systems, will be useful in the identification of inhibitor molecules that may prevent the function of key RNA or DNA helicase(s). The identification of inhibitors specific for these enzymes that are critical for the growth and replication of human pathogens will assist in the generation of new disease-specific therapies.

In order that this invention be more fully understood, the following examples are set forth. These examples are for the purpose of illustrating preferred embodiments of this invention, and are not to be construed as limiting the scope of this invention in any way.

EXAMPLES

Material and Methods

Reagents. Sodium citrate buffers were prepared as 1.0 M stock solutions with citric acid and the pH was adjusted to the indicated value with concentrated NaOH. NH_2-activated 96 well plates were obtained from Costar. BS^3 and Surfact-Amps 20 were obtained from Pierce. Avidin and streptavidin were from Boehringer Mannheim. PBS was from JRH. Glycine was from Bio-Rad and prepared as a **1.0** M stock solution adjusted to pH 7.4 with NaOH prior to use. Acetylated BSA was obtained from GIBCO-BRL Life Sciences as 50 mg/mL stock solutions. Hepes was from Amersham, DTT was from Calbiochem-Novabiochem Corporation. ATP, GTP, and ADP were obtained in dry form and solutions of CTP and UTP (100 mM, pH 7) from Pharmacia Biotech. AMPPNP, AMPPCP, ATPγS, and ADPβS were from Boehringer Mannheim. Stock solutions of nucleoside phosphates were prepared at 100 mM final concentration and pH adjusted to 7.5 with concentrated NH_4OH. When nucleoside phosphates were used as potential helicase inhibitors, solutions were complexed with 1 mol of $MgCl_2$ per mol nucleoside phosphate to avoid altering the free Mg^{2+} levels in individual helicase reactions. ReadySafe liquid scintillation fluid and $\gamma[^{33}P]$ATP were from DuPont NEN Research Products.

Buffers. Buffer A was 20 mM NaCitrate, pH 4.0; 200 mM NaCl; and 1.0 mM EDTA. Buffer B was 20 mM Hepes, pH 7.5; 200 mM NaCl; 1.0 mM EDTA; 0.04% (v/v) Surfact-Amps 20. Buffer C was 40 mM Hepes, pH 7.5; 10% (v/v) glycerol; 5.5 mM $MgCl_2$; 4.0 mM ATP, 100 μg/mL BSA; 1.0 mM DTT. Buffer D was 20 mM Hepes, pH 7.5; 10% (v/v) glycerol; 200 mM NaCl; 0.1 mM EDTA; 0.1 mM EGTA; and 1.0 mM DTT.

Nucleic acid substrates. M13mp19 single-stranded DNA and the 68-mer oligonucleotide used to assemble the helicase substrates were prepared as follows: Double-tailed helicase substrates were assembled from ^{33}P labeled 68-mer oligonucleotides and M13mp19 single-stranded DNA exactly as described (9, 10). Following gel filtration the substrates were concentrated by diafiltration to a concentration of 1 to 3 mM in total nucleotide (0.15 to 0.5 μM in circular M13 molecules) (10). The specific activity of the helicase substrate was approximately 1.2×10^{14} cpm/mol nucleotide or 7.5×10^{17} cpm/mol M13mp19 molecules (e.g. 120 cpm/pmol nucleotide and 750 cpm/fmol M13 molecules, respectively). The specific activity of the single-stranded 68-mer used for oligonucleotide binding studies was 3.3×10^{17} cpm/mol 68-mer (e.g. 330 cpm/fmol).

Cells, viruses, proteins. The HSV-1 helicase-primase used in these studies was the three subunit holoenzyme comprised of the UL5, UL8 and UL52 proteins expressed in SF21 cells with previously described recombinant baculoviruses (10, 11). The recombinant enzyme was isolated and stored as described, stock solutions were at a concentration of 1.0 mg/mL (3.4 μM) (10). Avidin and streptavidin were prepared as 1.0 mg/mL solutions in PBS and dialyzed against excess PBS prior to use.

Example 1

Preparation of plates/adsorption of helicase substrates. Activation of the amine-derivatized 96 well plates was with BS^3 under conditions described by Costar. After activation for 30 min with BS^3 (1 mg/mL in PBS, 250 μL/well), plates were washed three times with excess PBS and derivatized with 150 μL/well of either avidin (10 μg/mL in PBS), streptavidin (10 μg/mL in PBS), glycine (100 mM), or PBS alone. After a period of 1.0 hour at 37° C., of 1.0 M glycine was added to each well and incubation continued for an additional 30 minutes at room temperature. The activated, derivatized plates were then washed two times with PBS and two times with Buffer A. Helicase substrates were diluted with Buffer A to a final concentration of 12 μM in nucleotide (1.9 nM in circular molecules) for subsequent use, the 68-mer oligonucleotide was diluted to a concentration of 4.2 nM (in molecules) prior to use. In all cases, 100 μL of the individual nucleic acid solutions were used for adsorption to the solid supports for a period of 3 to 4 hours at room temperature with shaking in a 100% relative humidity environment. After allowing for nucleic acid adsorption, the plates were washed 3 times with Buffer B. When plates were analyzed for the ability to adsorb nucleic acid molecules, samples were eluted overnight by the application of 200 μL of a solution of 0.2 M NaOH containing 1.0% (w/v) SDS. Quantitation of the adsorbed samples was by scintillation

counting after mixing with 800 μL of liquid scintillation fluid. This process eluted ≧95% of the bound DNA (data not shown). When helicase assays were performed, avidin-derivatized helicase substrate-adsorbed plates were used immediately after washing with Buffer B and removal of residual liquid.

Example 2

Assembly of helicase assays. Solid phase helicase assays were assembled on ice with avidin-derivatized 96 well plates to which M13mp19/^{33}P-labeled 68-mer hybrid substrates had been adsorbed in Buffer A and then washed with Buffer B (see above). Reaction premixes were prepared on ice in Buffer C. The indicated amount of the HSV-1 helicase-primase per reaction performed was added to each premix as a 1/10th volume aliquot in Buffer D. Each enzyme-containing reaction mixture was then dispensed in triplicate to individual substrate-containing microtiter plate wells (100 μL/well). Helicase reactions were initiated by transfer of the assembled plate(s) onto the surface of a constant temperature water bath maintained at 36 degrees centigrade. After the indicated times either 70 μL samples were removed or the entire helicase assay plate was returned to ice. When helicase reactions were stopped by transfer to ice temperature, samples (70 μL) were again removed. Individual samples were quantitated by liquid scintillation counting after the addition of 800 μL of ReadySafe liquid. The means and standard errors of individual triplicate helicase assays were determined.

Example 3

Adsorption of helicase substrates and oligonucleotide reaction products is pH-dependent. The model DNA helicase substrate analyzed contained a radiolabeled oligonucleotide with 3' and 5' non-hybridizing sequences when annealed to M13mp19 single-stranded DNA (9). I examined the ability of polystyrene microtiter plates derivatized with various molecules to support the adsorption of assembled helicase substrates comprised of a ^{33}P-labeled 68-mer hybridized to M13mp19 single-stranded DNA or the labeled 68-mer oligonucleotide product of the helicase reaction by the manipulation of pH. Acidic pH favored the stabile interaction of nucleic acids with the plate surfaces (FIG. 2A). Maximal adsorption of the helicase substrate was observed between pH 3.25 and pH 4.0. Nearly two-fold more substrate was bound by avidin-derivatized surfaces under optimal conditions than surfaces that were streptavidin or glycine derivatized. Under all of the conditions described, once substrates were bound to the surfaces, interactions were not disrupted by transfer to neutral pH.

Surfaces that were cytochrome C-derivatized behaved similarly to streptavidin or glycine-derivatized surfaces; HSV-1 SSB-coated surfaces were found to adsorb little of the input DNA at all pH values examined (data not shown). The adsorptive properties of the avidin-derivatized surfaces were found not as strictly dependent on the pH used for immobilization of the helicase substrates when compared to the other surfaces examined. Optimal substrate binding was still in the acidic pH range indicated above (FIG. 2A). The maximum amount of substrate was bound in the presence of 200 mM NaCl under the adsorption conditions examined. The amount of substrate bound at optimum NaCl concentrations was approximately twice the amount bound in Buffer A without NaCl and 1.5 times the amount bound in Buffer A with the NaCl concentration increased to 1.0 M (data not shown).

The helicase reaction product, the radiolabeled 68-mer oligonucleotide, was also found to stably interact with derivatized surfaces at acidic pH (FIG. 2B). The optimum pH range for binding of the radiolabeled 68-mer was more acidic when compared to the M13mp19/68-mer hybrid. Under optimal conditions, avidin-derivatized surfaces bound less of the 68-mer when compared to the other surfaces examined. Moreover, there was little detectable binding of helicase reaction products at neutral pH values; typical conditions used in helicase reactions. This implied that under conditions used in duplex nucleic acid unwinding assays, the released radiolabeled product of the helicase reaction, the 68-mer, would remain in solution for detection.

Example 4

Immobilized duplex DNA molecules are substrates for the HSV-1 helicase-primase. To characterize the ability of the HSV-1 helicase-primase to unwind adsorbed helicase substrate increasing amounts of the HSV-1 helicase-primase were added to M13mp19/^{33}P-labeled 68-mer coated wells (FIG. 3). A linear dependence between the addition of the HSV-1 helicase-primase and the amount of product released from adsorbed surfaces was found. Moreover, the catalytic release of helicase reaction products displayed an absolute dependence on the addition of ATP. The time dependence of the helicase reaction was also examined using optimal enzyme concentrations (FIG. 4). Release of the 68-mer helicase reaction product was linear to about 30 min. After 30 min, there was a notable decrease in the rate of product release in the helicase reactions. This may be due to time-dependent denaturation of the helicase enzyme. Alternatively, the products of the helicase reaction may be partially depleted or may act as competitive inhibitors to prevent further progress of the unwinding reaction (9).

Example 5

Analysis of potential nucleoside-based inhibitors of the HSV-1 helicase-primase holoenzyme. To display the utility of the solid phase helicase assay, I analyzed several commonly available nucleotides for inhibitory activity against the HSV-1 helicase-primase holoenzyme. AMPPCP, AMPPNP, ADPβS, ADP, or ATPγS were titrated in the assays designed to detect inhibitor activity (FIG. 5). AMPPCP and AMPPNP were not effective inhibitors of the helicase activity of the HSV-1 helicase-primase. ADPβS inhibited activity by approximately 50% at the highest concentration analyzed (6.0 mM). ADP and ATPγS were the most effective enzyme inhibitors. I found concentrations of 1.5 to 2.0 mM ADP and less than 0.5 mM ATPγS capable of inhibiting the helicase activity of the HSV-1 helicase-primase by at least 50%.

The ability of ADP to inhibit the helicase of the HSV-1 helicase-primase was analyzed further by examining helicase reactions driven by nucleoside triphosphates other than ATP (FIG. 6). GTP was nearly as effective at promoting helicase-mediated unwinding as ATP. CTP and UTP were less effective at promoting the helicase reaction; CTP was approximately half as effective as ATP and UTP slightly less effective than CTP. When nucleoside triphosphates other than ATP were used to drive helicase reactions, sensitivity to ADP inhibition was enhanced. GTP, CTP and UTP-driven helicase reactions were all inhibited to a greater extent by ADP when compared to the ATP-driven reaction. Essentially, the better the nucleoside triphosphates supported helicase activity, the less sensitive the activity was to ADP inhibition. This supports the observation that the

mechanism by which nucleoside inhibitors of helicase activity mediate inhibition is competitive with respect to nucleoside triphosphate binding.

References Cited

1. Baker, T. A. and Kornberg, A. (1992) DNA Replication, Second Edition, W. H. Freeman and Company, New York.
2. Abdel-Monem M., Durwald, H., and Hoffmann-Berling, H. (1976) *Eur. J. Biochem.* 65, 441–449.
3. Venkatesan, M., Silver, L. L., and Nossal, N. G. (1982) *J. Biol. Chem.* 257, 12426–12434.
4. Matson, S. W., Tabor, S., and Richardson, C. C. (1983) *J. Biol. Chem.* 258, 14017–14024.
5. Helicase [^{3}H]Scintillation Proximity Assay Enzyme Assay System, Product Specification Document (1996) Amersham Life Science, Arlington Heights, Ill.
6. Houston, P., and Kodadek, T. (1994) *Proc. Natl. Acad. Sci. USA* 91, 5471–5474.
7. Raney, K. D., Sowers, L. C., Millar, D. P., and Benkovic, S. J. (1994) *Proc. Natl. Acad. Sci. USA* 91, 6644–6648.
8. Eggleston, A. K., Rahim, N. A., and Kowalczykowski, S. C. (1996) Nucleic Acids Res. 24, 1179–1186.
9. Crute, J. J., Mocarski, E. S., and Lehman, I. R. (1988) *Nucleic Acids Res.* 16, 6585–6596.
10. Dracheva, S., Koonin, E. V., and J. J. Crute. Identification of the primase active site of the herpes simplex virus type 1 helicase-primase. (1995) *J. Biol. Chem.* 270: 14148–14153.
11. Dodson, M. S., Crute, J. J., Bruckner, R. C., and Lehman, I. R. (1989) *J. Biol. Chem.* 264, 20835–20838.

While I have described a number of embodiments of this invention, it is apparent that my basic constructions may be altered to provide other embodiments that utilize the products and processes of this invention. Therefore, it will be appreciated that the scope of this invention is to be defined by the appended claims, rather than by the specific embodiments which have been presented by way of example.

I claim:

1. A method for measuring the helicase inhibiting ability of a test substance comprising the steps of:

(a) immobilizing on a solid support under acid pH conditions a helicase substrate comprising a single stranded nucleic acid polymer hybridized to a labeled helicase reaction product, wherein the labeled helicase reaction product is detectable in solution by helicase activity;

(b) contacting the immobilized helicase substrate with a test substance to produce a reaction premix;

(c) contacting under neutral or slightly alkaline pH conditions the reaction premix of step (b) with a helicase capable of releasing labeled helicase reaction product into solution by helicase activity; and

(d) measuring the amount of labeled helicase reaction product released into solution as a function of time.

2. The method according to claim 1, wherein the labeled helicase reaction product comprises a hybridizing nucleic acid sequence optionally flanked by non-hybridizing sequences on its 5' and 3' end.

3. The solid phase helicase assay according to claim 2, wherein the hybridizing nucleic acid sequence comprises from 10–50 nucleotides and each of the non-hybridizing sequences comprises from 10–80 nucleotides.

4. The method according to claim 1, wherein the solid support is a protein-derivatized polystyrene microtiter well plate.

5. The method according to claim 4, wherein the protein is avidin.

6. The method according to claim 1, wherein the labeled helicase reaction product is radiolabeled.

7. The method according to claim 1, wherein the helicase is a herpes virus helicase-primase.

8. The method according to claim 7, wherein the herpes virus helicase-primase is the HSV-1 helicase-primase.

9. The method according to claim 8, wherein the model helicase substrate is M13mp19 single-stranded DNA hybridized with a ^{33}P-labeled helicase reaction product.

10. The solid phase helicase assay according to claim 1, wherein in step (a) the helicase substrate is immobilized on a solid support under acid pH of about 3.25 to 4.0.

* * * * *

Detailed Description Text - DETX (28):

The assays and methods of this invention envision the use of RNA-, DNA - or mixed RNA/DNA model helicase substrates. For instance, the extended single-stranded nucleic acid polymer and labeled helicase reaction product may be made of either RNA or DNA . Furthermore, the extended single-stranded nucleic acid polymer may be made of RNA, while the labeled helicase reaction product may be made of DNA (or visa versa). Such DNA/DNA, DNA /RNA, RNA/DNA and RNA/RNA combinations will allow for the study of all types of helicases, including prokaryotic replicative helicases (such as the dnaB protein of E. coli) and various eukaryotic helicases. Although the examples described herein utilize the HSV-1 helicase-primase and a DNA/DNA model helicase substrate, it should be recognized that any helicase may be used in the assays and methods of this invention with a model helicase substrate appropriate for that helicase.

US005741638A

United States Patent [19]

Yamane

[11] **Patent Number: 5,741,638**

[45] **Date of Patent: Apr. 21, 1998**

[54] **MICROTITER WELL FOR DETECTING NUCLEIC ACID**

[75] Inventor: **Akio Yamane**, Kouda-Cho, Japan

[73] Assignee: **Wakunaga Seiyaku Kabushiki Kaisha**, Osaka-Fu, Japan

[21] Appl. No.: **358,995**

[22] Filed: **Dec. 19, 1994**

Related U.S. Application Data

[63] Continuation of Ser. No. 4,572, Jan. 14, 1993, abandoned, which is a continuation-in-part of Ser. No. 722,673, Jun. 28, 1991, abandoned.

[30] **Foreign Application Priority Data**

Jun. 28, 1990 [JP] Japan 2-170684

[51] **Int. Cl.**[6] **C12Q 1/68**; A61L 3/00

[52] **U.S. Cl.** **435/6**

[58] **Field of Search** 435/6; 935/77, 935/78; 427/2, 58; 422/102; 436/809

[56] **References Cited**

PUBLICATIONS

Cook et al., Nucl. Acid. Res. 16:4077–4095 (1988) "Synthesis and Hybridization of a Series . . . ".

Nagata et al., FEBS 183: 379–382 (1985) "Quantification of Picogram Levels of Specific DNA . . . ".

Rosmvssen et al., Analytical Biochem 198:138–142 (1991) "Covalent Immobilization of DNA . . . ".

Murray et al., Mol. Biochem. Parasitol 30:209–216 (1988) "Cloning and Characterization of a Species–spefic . . . ".

Urdea et al, Nucleic Acid Res 16: 4937–4956 (1988).

Primary Examiner—Eggerton A. Campbell

Attorney, Agent, or Firm—Wenderoth, Lind & Ponack

[57] **ABSTRACT**

A microtiter well in which a single stranded nucleic acid including a plurality of sequences specifically hybridizalbe with a target nucleic acid is immobilized is disclosed. The single stranded nucleic acid is derived from a phage or a phage-plasmid. The microtiter well enables a target nucleic acid to be specifically detected with a high sensitivity and high efficiency and further enables the detection procedure to be automated.

4 Claims, 6 Drawing Sheets

1623

1–6, 9,

12–18

20

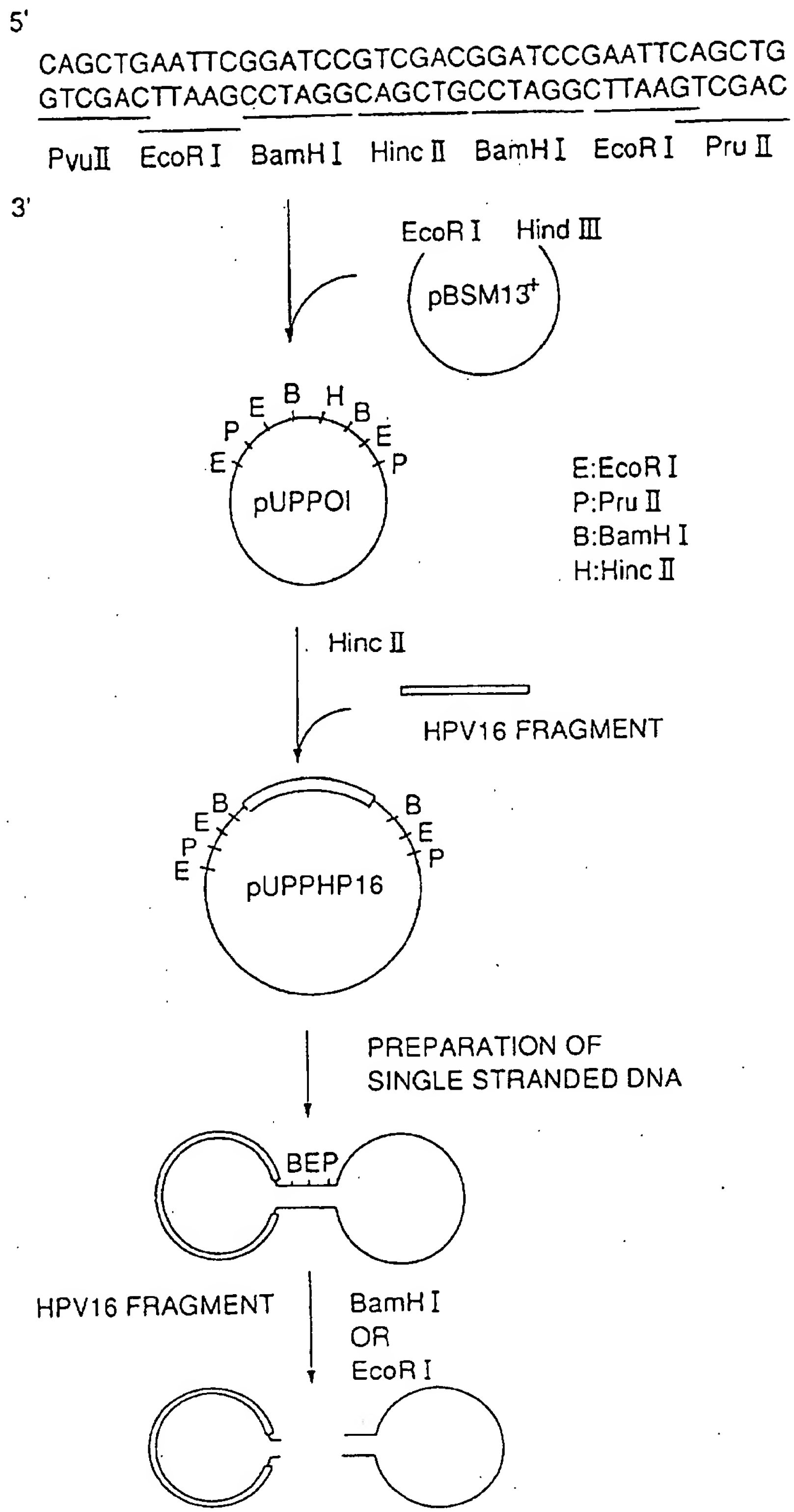
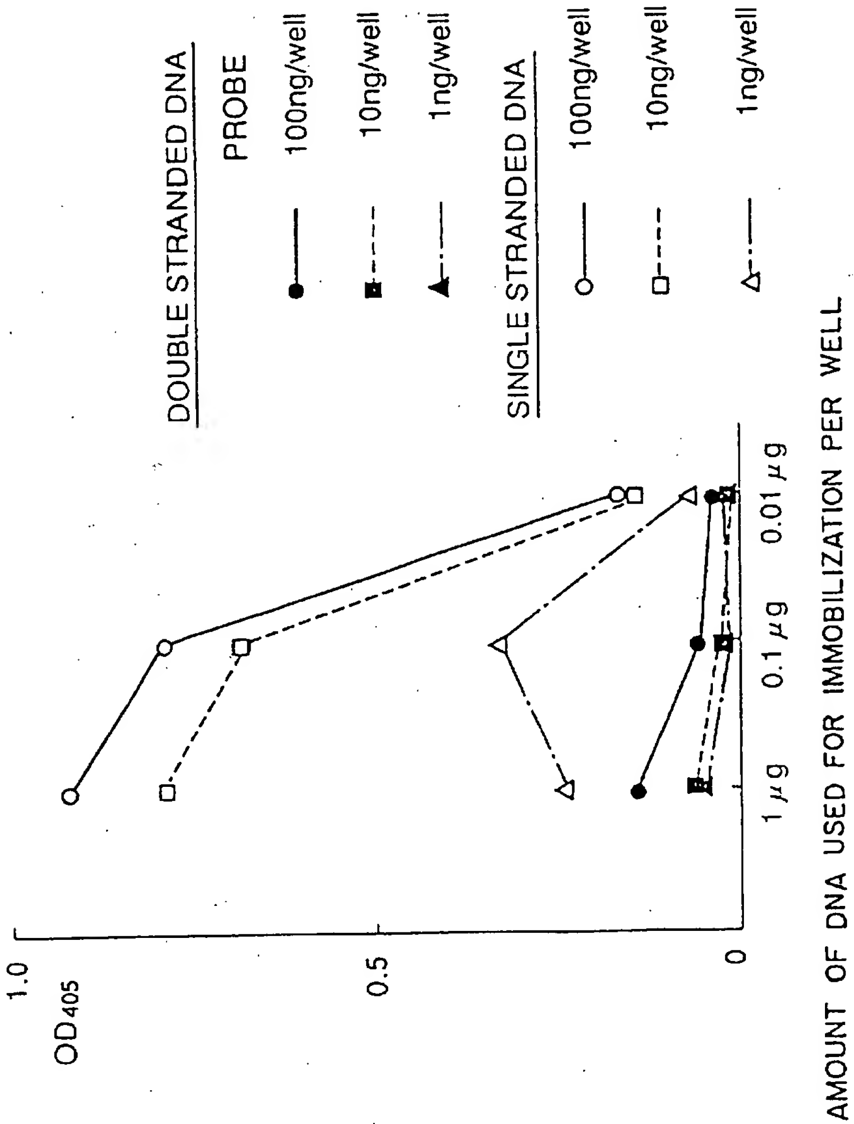

5'
CAGCTGAATTCGGATCCGTCGACGGATCCGAATTCAGCTG
GTCGACTTAAGCCTAGGCAGCTGCCTAGGCTTAAGTCGAC
PvuII
EcoR I
BamH I
Hinc II
BamH I
EcoR I
Pru II
3'
EcoR I
Hind III
pBSM13+
E
P
E
B
H
B
E
P
pUPPOI
E:EcoR I
P:Pru II
B:BamH I
H:Hinc II
Hinc II
HPV16 FRAGMENT
B
E
P
E
B
E
P
pUPPHP16
PREPARATION OF
SINGLE STRANDED DNA
BEP
HPV16 FRAGMENT
BamH I
OR
EcoR I

FIG. 1



DOUBLE STRANDED DNA
PROBE
100ng/well
10ng/well
1ng/well
SINGLE STRANDED DNA
100ng/well
10ng/well
1ng/well
OD405
1.0
0.5
0
1 μg
0.1 μg
0.01 μg
AMOUNT OF DNA USED FOR IMMOBILIZATION PER WELL

FIG. 2

5'
CACCTGACTCCTG$\boxed{A}$GGAGAAGTCTGCCGTT βA
GTGGACTGAGGAC$\boxed{T}$CCTCTTCAGACGGCAA
3'

5'
CACCTGACTCCTG$\boxed{T}$GGAGAAGTCTGCCGTT βS
GTGGACTGAGGAC$\boxed{A}$CCTCTTCAGACGGCAA
3'

FIG. 3

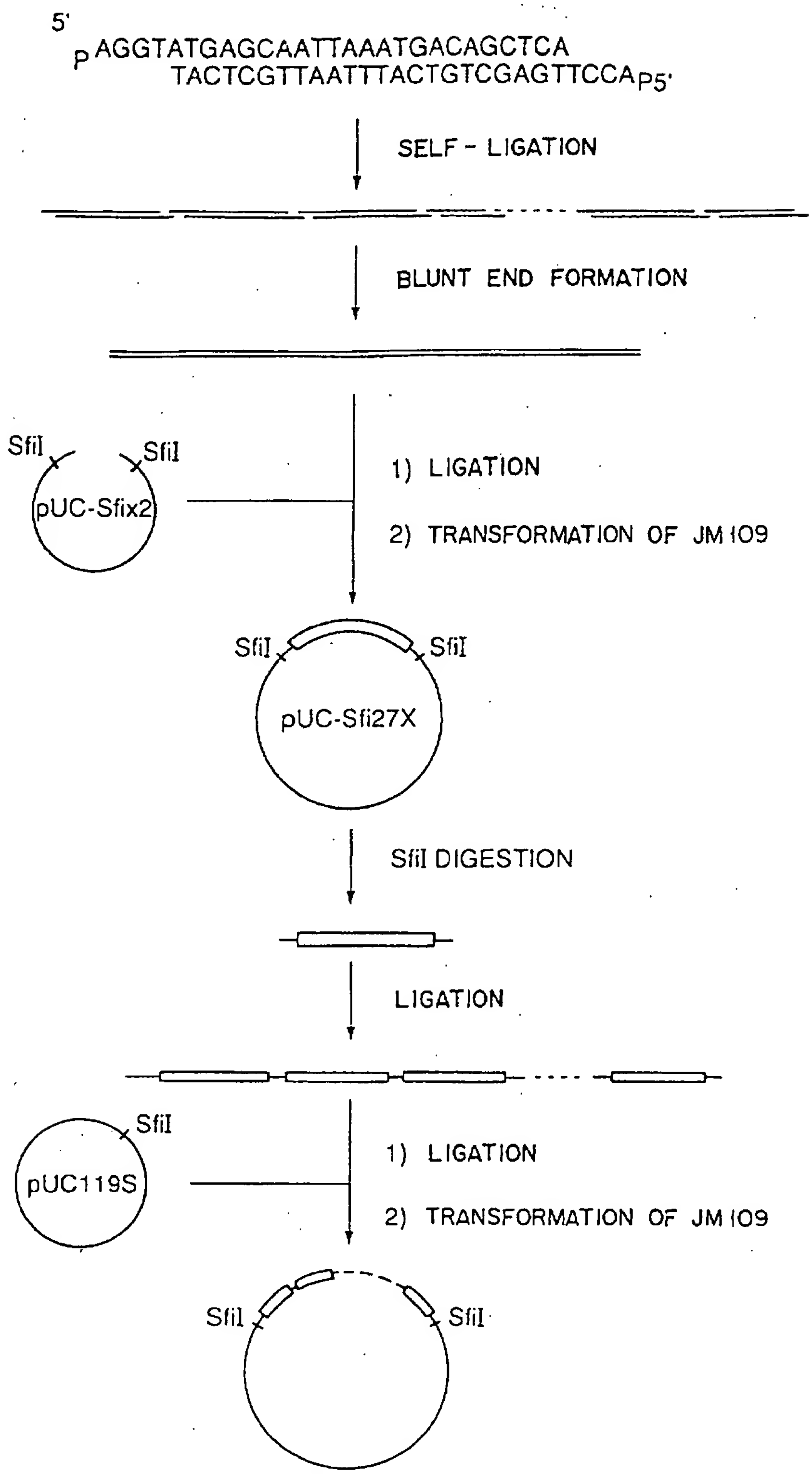

5'
p AGGTATGAGCAATTAAATGACAGCTCA
TACTCGTTAATTTACTGTCGAGTTCCA p 5'
SELF - LIGATION
BLUNT END FORMATION
SfiI
SfiI
pUC-Sfix2
1) LIGATION
2) TRANSFORMATION OF JM109
SfiI
SfiI
pUC-Sfi27X
SfiI DIGESTION
LIGATION
SfiI
pUC119S
1) LIGATION
2) TRANSFORMATION OF JM109
SfiI
SfiI

FIG. 4

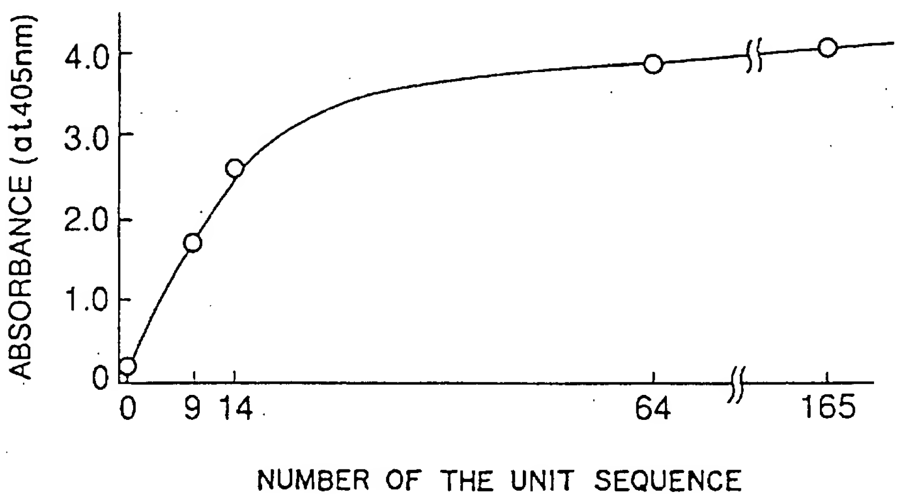

ABSORBANCE (at405nm)
4.0
3.0
2.0
1.0
0
0
9
14
64
165
NUMBER OF THE UNIT SEQUENCE

FIG. 5

```
               1        10        20        30
DRB1*0101      GGGGACACCCGACCACGTTTCTTGTGGCAG
DRB1*1201      ---------A--------------GA-T-C
DRB1*0901      ******************************
DRB5*0101      ------------------------CA----
                                       CAGCAG

                        40        50        60
DRB1*0101      CTTAAGTTTGAATGTCATTTCTTCAATGGG
DRB1*1201      TC--C-GG---G---T--------------
DRB1*0901      *********************-----C---
DRB5*0101      GA-----A---G--------------C---
               GATAAGTA
                Probe A

                        70        80        90
DRB1*0101      ACGGAGCGGGTGCGGTTGCTGGAAAGATGC
DRB1*1201      -----------------A-----G---CA-
DRB1*0901      ----------------AT---C-C---G--
DRB5*0101      -----------------C---C-C---GA-
                            GGTTACTGGAGAG
                               Probe B
                             GTATCTGCACAGAGG
                                 Probe C
```

-: same as that of the uppermost sequence
*: unknown

FIG. 6

MICROTITER WELL FOR DETECTING NUCLEIC ACID

This application is a continuation of now abandoned application Ser. No. 08/004,572, filed Jan. 14, 1993 abandoned, which is a continuation-in-part application of Ser. No. 07/722,673 filed Jun. 28, 1991 abandoned.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to a microtiter well for detecting a nucleic acid in a biological sample. More particularly, this invention relates to a microtiter well which enables a target nucleic acid to be rapidly detected and the detection procedure to be automated.

2. Description of the Related Art

Hybridization processes for detecting a specific sequence of a nucleic acid include a solid-liquid hybridization process wherein nucleic acids adsorbed on a solid support are hybridized with nucleic acids contained in a solution; and a liquid-liquid hybridization process wherein target nucleic acids and nucleic acid probes contained in a solution are hybridized with each other (Anal. Biochem., 169, 1–25 (1988)). Up to now, these processes have been improved in various ways for the purpose of more easily, more rapidly and more sensitively detecting a target nucleic acid (Anal. Biochem., 169, 1–25 (1988)).

Among them, there is a proposal on a process wherein a microtiter well used in the field of immunoassay is utilized for automating the procedure of detecting a target nucleic acid (Japanese Patent Laid-Open Publication No. 219400/86). In this process, after double stranded DNA in a sample is converted to a single stranded form, the single stranded nucleic acids are immobilized through a nonspecific adsorption on a microtiter well under such a condition that complementary strands are present. Due to the inherent characteristic of DNA, some of the single stranded nucleic acids return to a double stranded form during the immobilization process and the subsequent hybridization process, so that in an actual hybridization the amount of the hybridizable DNA appears to be substantially smaller than that of immobilized DNA. Furthermore, in this process, the amount of DNA immobilized is relatively small. This renders the process unsatisfactory from the practical viewpoint.

Immobilization of polythymidylic acids on a microtiter well has been proposed as a capturing probe for sandwich hybridization process (Molecular and Cellular Probes, 3, 189–207 (1989)). This process is advantageous in that the amount of DNA immobilized is much larger than that in the case of a mixed base sequence of DNA by virtue of immobilization of a polymer of thymidylic acid having a higher reactivity than that of other nucleic acid bases in a photoreaction. The application of immobilized polythymidylic acids, however, is limited to a sandwich hybridization, so that the process suffers from the disadvantages of a complicated procedure and a lowered sensitivity inherent in the sandwich hybridization.

A nucleic acid amplification process called the PCR (Polymerase Chain Reaction) was developed in recent years. It is the epoch-making process which can amplify a minute amount of a certain gene by one hundred thousand times or more in a short time (U.S. Pat. No. 4,683,195). A proposal has been made on a process for detecting a mutation of human gene through the utilization of nucleic acids amplified by PCR (Proc. Natl. Acad. Sci., U.S.A., 86, 6230–6234 (1989)). The proposed process is called "reverse dot hybridization" and characterized by chemically synthesized oligonucleotides complementary to a base sequence to be detected and adding polythymidylic acid to the oligonucleotides by an enzymatic reaction to facilitate the immobilization of the oligonucleotide on a nylon membrane. This process has the advantage of the oligonucleotide being efficiently immobilized on a nylon membrane, but is disadvantageous in that the process in which use is made of a nylon membrane, as such, is unsuitable for automation of the detection procedure and the preparation of the immobilized probes are unsuitable for mass production.

Although the above processes have advantages in some results, they have respective drawbacks and no process capable of realizing a satisfactory simplicity, rapidity and high sensitivity has been developed in the art.

SUMMARY OF THE INVENTION

The present inventor has now found that a single stranded nucleic acid derived from a phage or a composite vector comprising a phage and a plasmid can be immobilized through a nonspecific adsorption in a microtiter well, and that the microtiter well in which the single stranded nucleic acids are immobilized can be used for detecting a target nucleic acid in a biological sample with a high sensitivity.

Accordingly, an object of the present invention is to provide a microtiter well capable of detecting a target nucleic acid in a biological simple, rapid and highly sensitive manner.

Another object of the present invention is to provide a microtiter well which enables a detection procedure to be automated.

According to a first aspect of the invention, there is provided a microtiter well for detecting the presence or absence of a target nucleic acid comprising a well in which a single stranded nucleic acid including a plurality of sequences specifically hybridizable with the target nucleic acid is immobilized, the single stranded nucleic acid being derived from a phage or a phage-plasmid.

The microtiter well according to the present invention enables a target nucleic acid to be specifically detected with a high sensitivity and high efficiency and further enables the detection procedure to be improved in simplicity and rapidity and to be automated.

BRIEF DESCRIPTION OF THE DRAWINGS

The aforesaid and other objects and features of the present invention will now become apparent from the following detailed description with reference to the accompanying drawings, in which:

FIG. 1 is a diagram showing the construction of an improved vector for preparing a linear single stranded DNA and the preparation of the single stranded DNA;

FIG. 2 is a graph showing the results of a comparison of the hybridization efficiency on a single stranded DNA immobilized on a microtiter well with that on a double stranded DNA immobilized on a microtiter well;

FIG. 3 is a diagram showing nucleic acid sequences inserted into a vector for the preparation of an immobilized single stranded DNA in the detection of point mutation of β-thalassemia gene;

FIG. 4 is a diagram showing the preparation of a plasmid vector containing repetition of a oligonucleotide unit complementary to a human papilloma virus 16 gene;

FIG. 5 is a graph showing the relationship between the number of oligonucleotide units of an immobilized probe and the sensitivity; and

FIG. 6 is a diagram showing sequences of respective types of HLA-DB genes and a sequence of a probe used in Example 11.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The term "target nucleic acid to be detected" used herein is intended to mean a nucleic acid containing a specific base sequence to be detected and may be any of DNA and RNA. The nucleic acids to which the present invention is applicable can be prepared from every form of life including bacteria, viruses and higher plants and animals. The nucleic acids may be in a purified form or in an unpurified form.

In a nucleic acid detection process using a microtiter well of the present invention, not only a nucleic acid obtained from the above-described life but also a synthesized nucleic acid corresponding to a nucleic acid derived from the specimen or a synthesized nucleic acid complementary to the nucleic acid may be used as the target nucleic acid to be detected. Therefore, in the present invention, the "target nucleic acid" includes a nucleic acid in a biological sample to be detected, a synthesized nucleic acid corresponding to the nucleic acid in the biological sample and a synthesized nucleic acid complementary to the nucleic acid in the biological sample.

According to the present invention, single stranded nucleic acids specifically hybridizable with a target nucleic acid is immobilized on a microtiter well, preferably a polystyrene microtiter well. In the microtiter well, the immobilized single stranded nucleic acid are under such a condition that the strands complementary to the single stranded nucleic acids are absent.

In the present invention, a single stranded nucleic acid derived from a phage or a composite vector comprising a phage and a plasmid which contain a gene to be detected is used. Preferred examples of the phage and the composite vector include M13 phage, pUC118, pBSM13(±), pUCf1 (Methods in Enzymology, 153, 3–34, (1978)), pGEM-325 (±), pGEM-525(±), pGEM-725(±), pGEM-925(±), pGEM-1125(±), pGEM-1352(±), pGEMEX-1, pGEMEX-2, pSELECT-1 (available from PROMEGA), pT3/T7-3, pYEUra3 (available from CLONTECH), pKMN(±) (available from TOYOBO CO., LTD.), M13mp18/19, M13tu18/19, pUC118, pUC119, pTU118, pTU119, pTWU228, pTWU229 (available from TAKARA SHUZO CO., LTD.), pSL1180, pSL1190, pTZ18R, pTZ19R, pT7T3u18U, pT7T3u19U (available from PHARMACIA), pBluescript II SK(±), pBluescript II KS(±), pBc SK(±), pSc KS(±), pSluescript SK(±), pBluescript KS(±) and pBS (±) (available from STRATAGENE). Although the single stranded nucleic acid which is derived from the phage or the composite vector is immobilized in a circular form as it is, the single stranded nucleic acid can be digested to provide a linear form, preferably 1 k bs to 8 k bs fragment, and then immobilized.

According to another aspect of the present invention, the single stranded nucleic acid to be immobilized preferably contains a plurality of sequences hybridizable with a target nucleic acid. The presence of many sequences hybridizable with a target nucleic acid in a single stranded nucleic acid to be immobilized can shorten the time necessary for the hybridization, so that a gene can be rapidly detected.

The single stranded nucleic acid containing a plurality of sequences hybridizable with a target nucleic acid include can be prepared by introducing a plurality of sequences hybridizable with a target nucleic acid into the above-described phage DNA or a composite vector comprising a phage and a plasmid and producing a single stranded nucleic acid therefrom. In particular, it is preferred to use a single stranded nucleic acid prepared from a vector containing 5 to 200 copies of a sequence hybridizable with a target nucleic acid.

When a point mutation or the like in a nucleic acid sequence in a sample should be detected by hybridization, it is preferred that a sequence hybridizable with a target nucleic acid is relatively short.

The use of single stranded nucleic acids containing a plurality of sequences hybridizable with a target nucleic acid is advantageous in that the sequence hybridizable with the target nucleic acid can be immobilized in a relatively large amount.

It is known that an oligonucleotide immobilized on a solid support has a poor hybridization efficiency because the oligonucleotide immobilized on the solid support has a portion which participates in the immobilization on the support and cannot involve in hybridization. The hybridization efficiency of the immobilized oligonucleotides is, in general, lower than that attained in the solution. When use is made of single stranded nucleic acids prepared from the above-described vector containing a plural number of a unit sequence hybridizable with a target nucleic acid, a number of the unit sequences which do not involve in the immobilization on a solid support appear to be present. Thus, the lowering in the hybridization efficiency derived from the immobilization is advantageously small.

When labelling of a target nucleic acid is conducted by use of an elongation reaction or an amplification reaction and the labelled product to be detected, the single stranded nucleic acid to be immobilized is preferably less homologous to primers utilized for the elongation reaction or the amplification reaction. For example, in the PCR process, in many cases, the primer used in the gene amplification remains in the solution after the amplification reaction. In this case, it is necessary to select the sequence of the single stranded nucleic acid in such a manner that the primers do not hybridize with the single stranded nucleic acid. This is true of the other gene amplification methods.

It was surprising that the single stranded nucleic acids derived from the phages or the composite vectors comprising a phage and a plasmid which contain a gene to be detected can be directly immobilized on a microtiter well through a nonspecific absorption efficiently. In particular, the single stranded nucleic acids having 1 k bs to 8 k bs size can be directly immobilized on a microtiter well in a large amount. Thus, it is preferred to use the single stranded nucleic acids having 1 k bs to 8 k bs size. In addition, a microtiter well on which the single stranded nucleic acids having 1 k bs to 8 k bs size are immobilized ensures a detection of a target nucleic acid in a high sensitivity. The adsorption of the single stranded nucleic acid on the microtiter well can be preferably enhanced by irradiation of ultraviolet light or by adding $MgCl_2$ (Japanese Patent Laid-Open Publication No. 219400/1986).

In the nucleic acid detection process using a microtiter well according to the present invention, a target nucleic acid to be detected may be labelled. Examples of the labelling method include (1) a method wherein a label is directly introduced into a target nucleic acid, (2) a method wherein a nucleic acid corresponding to a target nucleic acid or a nucleic acid complementary to the target nucleic acid is synthesized with a labelled oligonucleotide primer and (3) a method wherein a nucleic acid corresponding to a target

nucleic acid or a nucleic acid complementary to the target nucleic acid is synthesized with an oligonucleotide primer in the presence of a labelled nucleotide unit.

A method wherein a biotin derivative is introduced into a target nucleic acid by a photoreaction and a detection is conducted through an enzyme conjugated streptavidin (Nucleic Acids Res., 13, 745 (1985)); and a method wherein a target nucleic acid is sulfonated and detected through an enzyme conjugated anti-sulfon antibody (Proc. Natl. Acad. Sci., U.S.A., 81, 3466–3470 (1984)) are preferred as the above method (1) wherein a label is directly introduced into a target nucleic acid from the viewpoints of simplicity and rapidity of the procedure.

An amplification of a specific nucleic acid sequence (BIO/TECHNOLOGY, 8, 291 (1990)) may be utilized for the above methods (2) and (3). The methods have drawn attention particularly in respect of the amplification of a target nucleic acid and further are of a high value in relatively simple labelling of a synthesized nucleic acid corresponding to a target nucleic acid or a synthesized nucleic acid complementary to the target nucleic acid. For example, in the PCR process (Science, 230, 1350–1354 (1985)), a labelled elongation product or amplification product can be prepared with a labelled primer or labelled mononucleotides. In the amplification method wherein Qβ replicase is utilized (BIO/TECHNOLOGY, 6, 1197 (1988)), a labelled elongation product or amplification product can be prepared with similarly labelled mononucleotides. Also in the nucleic acid amplification method other than described above, an elongation product or an amplification product can be labelled with labelled mononucleotides or labelled oligonucleotides incorporated by an elongation reaction or an amplification reaction. The method (2) is preferred in the present invention.

The label used herein may be radioactive or non-radioactive as far as this substance can be detected after the hybridization procedure. A non-radioactive label is preferred from the viewpoint of handleability, storage stability and disposal and because it can exhibit most efficiently the effect of the present invention.

Examples of the non-radioactive label include haptens, such as biotin, 2,4-dinitrophenyl group and digoxigenin, fluorescent substances such as fluorescein, rhodamine, tetramethylrhodamine, sulfo rhodamine, 7-nitrobenz-2-oxa-1,3-diazole (NBD) and dansyl group or chemiluminescent substances such as acridine. An oligonucleotide can be labelled with the substance by any of known means (Japanese Patent Laid-Open Publications No. 204200/84 and Japanese Patent Nos. 1651975 and 1706289). When labelled nucleotides are used, the labelling can be conducted by known means (Proc. Natl. Acad. Sci., U.S.A., 80, 4045 (1983) and Japanese Patent Laid-Open Publication No. 152364/88). Alternatively, a commercially available product may be utilized.

The target nucleic acid labelled as described above is hybridized with the single stranded nucleic acid immobilized in a microtiter well.

The hybridization conditions may be properly selected and determined according to a combination of the target nucleic acid with the immobilized single stranded nucleic acid. For example, the hybridization can be basically conducted in the same manner as that wherein the conventional membranes may be used (B. D. Hames and S. J. Higgins, Nucleic Acid Hybridization, A Practical Approach, IRL Press (1985)).

The washing procedure after the hybridization reaction as well can be conducted in the same manner as that of the conventional process. Because of simplicity, it is preferred to conduct the washing procedure under such a condition that excess reagents etc. can be removed at room temperature.

In the detection of a point mutation, the washing conditions should be carefully determined. It is also useful to utilize such a condition wherein the stability of the duplex depends upon only the length of the complementary strand but does not upon the base composition of the complementary strand (Nucleic Acids Res., 16, 4637–4650 (1988)).

Simultaneously with or after the hybridization procedure described above, a target nucleic acid is detected through a label present in the target nucleic acid.

The detection procedure may be properly selected and determined according to the kind of the label present in the target nucleic acid.

When the label is directly detectable, that is, when the label is, for example, a radioisotope, a fluorescent substance or a dye, the detection procedure may be conducted in such a state that a labelled nucleic acid is bonded to a solid phase, or alternatively the detection procedure may be conducted by liberating the label into a solution in such a state that it is bonded to the nucleic acid or released from the nucleic acid and then conducting the detection according to the label. On the other hand, when the label is indirectly detectable, that is, when the label is a ligand capable of causing a specific binding reaction, such as biotin or hapten, the detection procedure can be conducted by a method commonly used in the detection of this type, that is, by use of an acceptor (for example, avidin or antibody) to which a label capable of directly generating a signal or an enzyme catalyzing a signal generating reaction has been bound. The acceptor may be previously added in the hybridization procedure. In this case, the binding process of the ligand to the acceptor can be conducted simultaneously with the hybridization step with the result that the whole process can be simplified.

It is advantageous that the microtiter wells according to the present invention enable a detection of a plural of biological samples simultaneously. It is also advantageous that the microtiter well according to the present invention enable the detection procedure to be automated.

EXAMPLES

The present invention will now be described in more detail by way of the following examples, though it is not limited to these examples only.

The procedure of the genetic engineering techniques in the following examples was conducted according to Molecular Cloning, the 2nd edition (T. Maniatis et al., Cold Spring Harbor Laboratory Press (1989)). Oligonucleotide was prepared by use of a model 381 A automatic synthesizer manufactured by Applied Biosystems, Inc. and subjected to deprotection reaction and purification before use by the conventional procedure (Oligonucleotide Synthesis, IRL Press (1984)). The biotin-labelled oligonucleotide was prepared by adding Aminolink II (trademark) (manufactured by Applied Biosystems, Inc.) to oligonucleotide in the final stage of the synthesis of the oligonucleotide to introduce an amino group into the oligonucleotide, and reacting the oligonucleotide with biotin succinimide ester according to the method described in U.S. Patent No. 4,849,336.

Example 1

Preparation of single stranded DNA for immobilization

A linear single stranded DNA was prepared by a modified method of Messing et al. (Methods in Enzymology, 101, Part

C, 20 (1983)). A chemically synthesized DNA fragment shown in FIG. 1 (SEQ ID NO. 1) was inserted into between EcoRI and Hind III sites of plasmid pBSM13+ (manufactured by Stratagene Cloning Systems, Inc.) to prepare plasmid pUPPO1. Then, a 1.8 kb fragment containing genes E6 and E7 of human papilloma virus 16 was inserted into a Hinc II site of this plasmid to prepare plasmid pUPPHP16. *E. coli* NM522 was transformed with the plasmid, and a single stranded DNA was prepared by the conventional procedure with helper phage M13K07 (Methods in Enzymology, 153, 3–34 (1987)).

The single stranded DNA was cleaved by restriction enzyme EcoRI or BamHI for linearization.

Example 2

Immobilization of single stranded DNA in microtiter well

The single stranded DNA prepared in Example 1 was dissolved in a solution of 10 mM Tris.HCl, pH 7.6, and 1 mM EDTA to a concentration of 100 ng/μl and then mixed with a four-fold volume of H_2O and a five-fold volume of an immobilization buffer (1.5M NaCl, 0.3 M Tris.HCl, pH 8.0, 0.3M $MgCl_2$). The mixture was added to microtiter wells (Dynatech, Immulon 2, removawell strips, No. 011-010-6302) in an amount of 100 μl per well. The wells were covered and allowed to stand at 37° C. for 16 hr. Then, the liquid was removed, and the wells were air-dried at 37° C. for 30 min and subjected to light irradiation of 500,000 μJ through the use of Stratalinker (trademark) 2400 (manufactured by Stratagene Cloning Systems, Inc.). After the irradiation, the wells were washed three times with a washing buffer (1M NaCl, 2 mM $MgCl_2$, 0.1M Tris.HCl, pH 9.3, 0.1% Tween 20:200 μl). The wells were then sealed in a polyvinyl chloride bag and stored at 4° C.

Example 3

Hybridization in microtiter well and detection

A hybridization solution (5×SSC, 5×Denhardt's solution, 0.2% SDS, 200 μg/ml, salmon sperm DNA: 100 μl/well) was added to the microtiter wells in which the single stranded DNA containing a human papilloma virus 16 gene prepared in Example 2 had been immobilized, and further a dilution of biotin-labelled oligonucleotides (Bio-ATTGTAATGGGCTCTGTCCG, 20 ng/well (SEQ ID NO. 2) which are complementary to part of the human papilloma virus 16 gene was added thereto. The mixture was maintained at 55° C. for 30 min. The hybridization solution was removed, and the wells were washed three times with 2×SSC (200 μl/well). A streptavidin-alkaline phosphatase solution (prepared by subjecting a solution of streptavidin-alkaline phosphatase (Bethesda Research Laboratories, Inc.) to 1,000-fold dilution with 0.1M Tris.HCl, pH 7.5, 0.3M NaCl, 2 mM $MgCl_2$, 0.05% (v/v) Triton X-100) was added thereto (100 μl/well), and the wells were shaken at 23° C. for 10 min. The reaction mixture was removed from the wells, and the wells were washed three times with a washing solution (0.1M Tris.HCl, pH 7.5, 0.3M NaCl, 2 mM $MgCl_2$, 0.05% (v/v) Triton X-100: 200 μl/well). After the washing, a p-nitrophenyl phosphate solution (1M diethanolamine, pH 9.8, 0.5 mM $MgCl_2$: 4 mg/ml: 100 μl/well) was added to the wells and a reaction was allowed to proceed at 23° C. for one hr. Absorbance was then measured at 405 nm. The results are given in Table 1.

TABLE 1

Well No.	Absorbance (at 405 nm)
1	1.34
2	0.13
3	0.13

Well No. 1: the well in which the single stranded nucleic acid containing a human papilloma virus sequence was immobilized.
Well No. 2: the well in which the single stranded nucleic acid unrelated to a human papilloma virus sequence was immobilized.
Well No. 3: the well in which no DNA was immobilized.

In the table, the absorbance were corrected by subtracting the background at 405 nm.

Example 4

Effect of UV irradiation on immobilization of DNA in microtiter well

The plasmid DNA (pUPPHP16) and the single stranded DNA prepared therefrom prepared in Example 1 were linearized by cleaving with EcoRi and then immobilized in microtiter wells in the same manner as that of Example 2. In this case, when the double stranded DNA, i.e., pUPPHP16, was immobilized, heat denaturation was conducted before the double stranded DNA was mixed with the immobilization buffer. Then, two types of microtiter wells were prepared. Specifically, in one, UV irradiation was conducted in the same manner as that of Example 2, and in the other, no UV irradiation was conducted. The hybridization capability of the wells thus prepared was examined with the oligonucleotides, Bio-ATTGTAATGGGCTCTGTCCG (SEQ ID NO. 2), in the same manner as that of Example 3. The results are given in Table 2.

TABLE 2

Well No.	Absorbance (at 405 nm)
1	1.58
2	0.58
3	0.51
4	0.48

Well No. 1: the well in which the single stranded DNA was immobilized with UV irradiation.
Well No. 2: the well in which the single stranded DNA was immobilized without UV irradiation.
Well No. 3: the well in which the double stranded DNA was immobilized with UV irradiation.
Well No. 4: the well in which the double stranded DNA was immobilized without UV irradiation.

In the table, the absorbance were corrected by subtracting the background at 405 nm.

Example 5

Comparison of hybridization capability after immobilization in microtiter well of single stranded DNA with that of double stranded DNA

The hybridization capability after immobilization in a microtiter well of a single stranded DNA was compared with that of a denatured double stranded DNA. The single stranded DNA and the double stranded DNA were prepared in substantially the same manner as that of Example 4, but the single stranded DNA were immobilized in a cyclic form.

The DNA solution was added in an amount of 1 μg, 100 ng and 10 ng each per well for the immobilization in the same manner as that of Example 2. The hybridization capability of each well was examined by use of Bio-ATTGTAATGGGCTCTGTCCG (SEQ ID NO. 2) as a probe in the same manner as that of Example 3. The results were as shown in FIG. 2 (the absorbance at 405 nm were measured by a microplate reader).

Example 6

Labelling by gene amplification and detecting amplification product and point mutation

An oligonucleotide corresponding to codons 2 through 11 of a human β-globin was chemically synthesized and inserted into a HincII site of plasmid pUCf1 (see FIG. 3). The procedure was conducted for both a normal gene (βA) (SEQ ID NO. 3) and a point mutated gene (βS) (SEQ ID NO. 4) causative of β-thalassemia. Among the resultant clones, a clone capable of providing a sense single stranded DNA was selected, and the single stranded DNA was prepared therefrom. The single stranded DNA thus were immobilized in a cyclic form in microtiter wells according to the procedure described in Example 2.

Then, a gene amplification was conducted. The reaction was conducted through the use of GeneAmp (trademark) manufactured by Cetus Corporation according to the protocol by the manufacturer. 5' ACACAACTGTGTGTTCACTAGC (SEQ ID NO. 5) and biotinylated Bio-CAACTTCATCCACGTTCACC (SEQ ID NO. 6) were used as the primer, and 4.4 kb DNA fragments prepared from the plasmid DNA (pBR322-HβPst) by the digestion of Pst I (DNA, 3, 7–15 (1984)) was used as the template.

After the gene amplification, an aliquot of the reaction mixture was heat-denatured and subjected to hybridization and subsequent detection in the same manner as that of Example 3. The results are given in Table 3 (the absorbance at 405 nm were measured by a microplate reader).

TABLE 3

Well No.	Absorbance (at 405 nm)
1	0.432
2	0.288
3	0.037

well No. 1: the well in which the single stranded DNA containing A-gene was immobilized.
well No. 2: the well in which the single stranded DNA containing S-gene was immobilized.
well No. 3: the well in which the single stranded DNA unrelated to -globin gene was immobilized.

In the table, the absorbance were corrected by subtracting the background at 405 nm.

Example 7

Preparation of single stranded DNA containing repetition of unit sequence

A part of E7 gene of human papilloma virus 16 was chemically synthesized, and single stranded DNA containing repetition of a sequence of the synthesized oligonucleotides (the unit sequence) was prepared therefrom according to the method shown in FIG. 4 (SEQ ID NO. 7).

Briefly, two kinds of oligonucleotides shown in FIG. 4 were chemically synthesized, and the 5' terminal thereof was phosphorylated with polynucleotide kinase and ATP. Then, the two kinds of oligonucleotides were mixed with each other to form a double strand. The fragments of the double strand were linked to each other with T4 DNA ligase. After the ligation, blunt ends were formed by use of the Klenow fragment of *E. coli* DNA polymerase and four kinds of deoxynucleoside triphosphates. The resultant reaction product was electrophoresed on 6% polyacrylamide gel, and a portion corresponding to 270 bp was cut out to recover DNA.

Then, plasmid pUC-Sfix2 (Japanese Patent Laid-Open Publication No. 190194/90) was cleaved by BamHI, and the terminal 5' phosphate was removed with alkaline phosphatase. This product was ligated with T4 DNA ligase to the DNA recovered from the polyacrylamide gel. *E. coli* JM109 was transformed with the ligated product. An stable clone was selected with ampicillin marker. The plasmid pUC-Sfi27x thus obtained was cleaved by SfiI, and a portion containing repetition of the unit sequence was purified and recovered by electrophoresis. This fragment was self-ligated with T4 DNA ligase and inserted into the SfiI site of plasmid pUC119S (prepared by inserting SfiI linker (manufactured by Boehringer Mannheim; #909785) into the BamHi site of pUC119). Clones are different from each other in the number of the unit sequence depending upon the degree of self-ligation. In the present experiment, clones wherein the numbers of the unit sequence is 9, 14, 64 and 165 were obtained.

Example 8

Effect of probe into which oligonucleotide has been repeatedly inserted

Single stranded DNAs were prepared respectively from the clone prepared in Example 7 and a separately prepared clone having one unit sequence in the same manner as that of Example 1 and immobilized in a microtiter well in the same manner as that of Example 2.

Then, the following gene amplification was conducted by using the following two biotin-labelled primers:

Bio-GCAACCAGAGACAACTGATC (SEQ ID NO. 8)

Bio-ATTGTAATGGGCTCTGTCCG (SEQ ID NO. 2)

and a plasmid containing a E7 gene of papilloma virus 16 (HPV16) as a template.

GeneAmp (trademark) manufactured by Cetus Corporation were added to 10 ng of plasmid pUPPHP16 (see Example 1) and 100 ng of each primer according to the protocol by the manufacturer, and the total volume of the reaction solution was adjusted to 100 μl with water. The reaction solution was heated at 95° C. for 5 min in a thermal cycler manufactured by Perkin-Elmer Cetus Instruments, Inc. to denature DNA, and 2.5 units of AmpliTaq (trademark) was added thereto. The cycle of heating at 72° C. for 60 sec, at 94° C. for 30 sec and at 50° C. for 30 sec was repeated 30 times.

The reaction mixture was diluted with water and 5 μl of the diluent was heat-denatured and hybridized with a previously prepared microtiter wells on which the single stranded DNA containing a E7 gene sequences has been immobilized under the same condition as that described in Example 3, and the absorbance was measured.

The results are shown in FIG. 5. As is apparent from the drawing, when the number of the unit sequence was 64 or 165, the sensitivity was about 20 times that in the case where the number of the unit sequence was one.

Example 9

Detection of human papilloma virus 16 gene in rapid and simple manner

In order to simplify the hybridization process of the present invention further, studies were conducted on the composition of the hybridization solution, hybridization temperature and color development time. According to results of the studies, the detection of a human papilloma virus 16 gene was conducted under the following simplified conditions.

10 ng of DNA extracted from Caski cells as a positive sample and 10 ng of DNA extracted from human peripheral blood as a negative sample were each used as a template, and gene amplification was conducted by the PCR process in the same manner as that of Example 8. The resultant reaction mixtures were added to wells prepared in Example 8. In one well single stranded DNA wherein the number of repetitions of the unit sequence was one had been immobilized, and in the other well, single stranded DNA wherein the number of

repetitions of the unit sequence was 64 had been immobilized. Hybridization was conducted in 100 μl of a hybridization solution (5×SSC, 0.2% SDS) with 5 μl of the above-described PCR mixture at 37° C. for 30 min. After the hybridization solution was removed from the wells, the wells were washed three times with 200 μl of a washing solution (2×SSC).

A streptavidin-alkaline phosphatase solution (prepared by subjecting a solution of streptavidin-alkaline phosphatase (manufactured by Bethesda Research Laboratories, Inc.) to 1,000-fold dilution with 0.1M Tris.HCl, pH 7.5, 0.3M NaCl, 2 mM $MgCl_2$, 0.05% (v/v) Triton X-100) was added thereto 100 μl/well, and the wells were gently shaken at 23° C. for 10 min. After the solution was removed from the wells, the wells were washed three times with a washing solution (0.1M Tris.HCl, pH 7.5, 0.3M NaCl, 2 mM $MgCl_2$, 0.05% (v/v) Triton X-100: 200 μl/well). After the washing, 100 μl of p-nitrophenyl phosphate solution (1M diethanolamine, pH 9.8, 0.5 mM $MgCl_2$: 4 mg/ml) was added to the wells and an enzymatic reaction was allowed to proceed at 23° C. for 20 min. The absorbance was then measured at 405 nm. The results are given in Table 4. As is apparent from Table 4, even when the procedure was simplified, the positive sample could be significantly distinguished from the negative sample in the case of single stranded DNA wherein the number of the unit sequence was 64.

TABLE 4

	Absorbance (at 405 nm)	
Well No.	Positive sample (from Caski cell)	Negative sample (from normal human)
1	0.04	0.00
2	0.73	0.02

Well No. 1: the well in which the single stranded DNA containing one unit sequence was immobilized.
Well No. 2: the well in which the single stranded DNA containing 64 repeats of the unit sequence was immobilized.

In the table, the absorbance were corrected by subtracting the background at 405 nm.

Example 10

Detection of mutation of HLA-DRB gene

Unit sequences as shown in Table 5, i.e., probes 1 to 12, were repeatedly ligated in the same manner as that of Example 7 and immobilized in microtiter wells, respectively. The probes are completely matched to a gene sequence of genotypes as shown in Table 5. The unit sequences in the single stranded nucleic acids are repeated sixty times.

TABLE 5

Probe	Sequence	Genotype	SEQ ID NO.
1	CGG TTG CTG GAA AGA TGC	DR1	9
2	CAG CAG GAT AAG TAT GAG	DR2	10
3	GGC CGG GTG GAC AAC TAC	DR3	11
4	TTG GAG CAG GTT AAA CAT	DR4	12
5	T GAT GAG GAG TAC TGG AA	DR5(W11)	13
6	GG TTA CTG GAG AGA CAC T	DR5(W12)	14
7	TTC TTG GAG TAC TCT ACG	DR3,DR5,DR6,DR8	15
8	TGG CAG GGT AAG TAT AAG	DR7	16
9	A GAC AGG CGG GCC CTG GT	DR8	17
10	G TAT CTG CAC AGA GGC AT	DR9	18
11	TTG AAG CAG GAT AAG TTT	DR10	19
12	TGC AGA CAC AAC TAC GGG	nonspecific (all genotypes)	20

A gene amplification of HLA-DRB gene was conducted in the same manner as that of Example 8 by use of 1 μg of DNA extracted from human peripheral blood as a template and biotin-labelled primers as follows:

GLPDRB1 Bio-TTCTTCAATGGGACGGAGCG (SEQ ID NO. 21)

GAMPDRB1 Bio-GCCGCTGCACTGTGAAGCTCTC (SEQ ID NO. 22)

(GLPDRB1 is corresponding to amino acids 17 through 23 of HLA-DRB and GAMPDRB1 is corresponding to amino acids 87 to 94 of HLA-DRB, J. Exp. Med., 169, 2263–2267, 1989)

The gene amplification reaction cycle of heating at 94° C. for 30 sec, at 50° C. for 30 sec and at 72° C. for 60 sec was repeated 30 times. 5 μl of the reaction mixture was added to the microtiter wells on which probes 1 to 12 had been immobilized, and the hybridization was conducted at 60° C. for one hr and the enzymatic reaction was conducted at 23° C. for one hr. Other detailed procedure was as same as that of Example 3.

Absorbance measured at 405 nm are given in Table 6. According to the results, genotypes of the samples are determined as given in the table.

TABLE 6

Sample	1	2	3	4	5	6	7	8	9	10	11	12	Genotypes
a	1.69	1.03	0.12	0.16	0.12	0.15	0.13	0.18	0.19	>2	0.08	>2	DR1 DR9
b	0.06	>2	0.11	0.19	0.11	0.17	1.81	0.16	0.95	0.99	0.09	>2	DR2 DR8
c	0.06	0.23	1.51	>2	0.08	0.50	>2	0.12	0.16	0.09	0.06	>2	DR3 DR4
d	0.12	0.18	0.12	0.17	1.83	0.65	>2	0.20	1.15	0.11	0.09	>2	DR5(W11) DR8
e	0.07	0.17	0.14	0.13	0.09	>2	>2	0.16	>2	0.11	0.07	>2	DR5(W12) DR8
f	0.09	0.29	0.09	0.18	0.11	0.58	>2	>2	0.23	0.11	0.06	>2	DR6 DR7
g	0.28	0.27	0.08	0.19	0.10	0.15	>2	0.20	>2	0.11	>2	>2	DR8 DR10

Example 11

Detection of mutation of HLA-DRB gene

Short Oligonucleotides, i.e., Probes A (SEQ ID NO. 23), B (SEQ ID NO. 24) and C (SEQ ID NO. 25) shown in FIG. 6, are immobilized in microtiter wells in the same manner as that of Example 2. Long Oligonucleotides, i.e., single stranded nucleic acids containing probes A, B and C sequence repeated 60 times, were prepared and then immobilized in microtiter wells, respectively, in the same manner as that of Example 7. Probes A, B and C are completely matched to gene sequences of genotypes DRB5*0101(SEQ ID NO. 26), DRB1*1201 (SEQ ID NO. 27) and DRB1*0901 (SEQ ID NO.28), respectively.

A gene amplification of HLA-DRB gene was conducted in the same manner as that of Example 10 to prepare Sample 1, 2 and 3. The hybridization procedure was conducted in the same manner as that of Example 3. Absorbance measured at 405 nm are given in Table 6. Although the short oligonucleotides have nonspecificity to the samples, each of the long oligonucleotides has a definite specificity to the samples. According the absorbance obtained with the long oligonucleotides, genotypes of the samples are determined as given in the table.

TABLE 7

	Absorbance (at 405 nm)						
	Short Oligonucleotides			Long Oligonucleotides			
Sample	Probe A	Probe B	Probe C	Probe A	Probe B	Probe C	Genotype
1	0.193	0.139	0.180	2.520	0.216	0.238	DRB5*0101
2	0.112	0.104	0.268	0.859	0.176	1.998	DRB1*1201
3	0.212	0.204	0.160	0.311	1.695	0.249	DRB1*0901

The results demonstrate that the long oligonucieotides on the microtiter wells has an advantage of typing the HLA-DRB gene compared with the short oligonucleotides.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i i i) NUMBER OF SEQUENCES: 29

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: other nucleic acid

(i i i) HYPOTHETICAL:

(i v) ANTI-SENSE:

(v) FRAGMENT TYPE:

(v i) ORIGINAL SOURCE:
(A) ORGANISM:
(B) STRAIN:
(C) INDIVIDUAL ISOLATE:
(D) DEVELOPMENTAL STAGE:
(E) HAPLOTYPE:
(F) TISSUE TYPE:
(G) CELL TYPE:
(H) CELL LINE:
(I) ORGANELLE:

(v i i) IMMEDIATE SOURCE:
(A) LIBRARY:
(B) CLONE:

(v i i i) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT:
(B) MAP POSITION:
(C) UNITS:

(i x) FEATURE:
(A) NAME/KEY:
(B) LOCATION:
(C) IDENTIFICATION METHOD:
(D) OTHER INFORMATION: /note= "sequence shown in Fig. 1 in 5'-3'direction"

(x) PUBLICATION INFORMATION:
(A) AUTHORS:
(B) TITLE:
(C) JOURNAL:
(D) VOLUME:
(E) ISSUE:
(F) PAGES:
(G) DATE:
(H) DOCUMENT NUMBER:
(I) FILING DATE:
(J) PUBLICATION DATE:
(K) RELEVANT RESIDUES IN SEQ ID NO:

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CAGCTGAATT CGGATCCGTC GACGGATCCG AATTCAGCTG 40

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: other nucleic acid

(i i i) HYPOTHETICAL:

(i v) ANTI-SENSE:

(v) FRAGMENT TYPE:

(v i) ORIGINAL SOURCE:
(A) ORGANISM:
(B) STRAIN:
(C) INDIVIDUAL ISOLATE:
(D) DEVELOPMENTAL STAGE:
(E) HAPLOTYPE:
(F) TISSUE TYPE:
(G) CELL TYPE:
(H) CELL LINE:
(I) ORGANELLE:

(v i i) IMMEDIATE SOURCE:
(A) LIBRARY:
(B) CLONE:

(v i i i) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT:
(B) MAP POSITION:
(C) UNITS:

(i x) FEATURE:
(A) NAME/KEY:
(B) LOCATION:
(C) IDENTIFICATION METHOD:
(D) OTHER INFORMATION: /note= "having biotin at 5'end with a spacer"

(x) PUBLICATION INFORMATION:
(A) AUTHORS:
(B) TITLE:
(C) JOURNAL:
(D) VOLUME:
(E) ISSUE:
(F) PAGES:
(G) DATE:
(H) DOCUMENT NUMBER:
(I) FILING DATE:
(J) PUBLICATION DATE:
(K) RELEVANT RESIDUES IN SEQ ID NO:

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:2:

ATTGTAATGG GCTCTGTCCG 20

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: other nucleic acid

(i i i) HYPOTHETICAL:

(i v) ANTI-SENSE:

(v) FRAGMENT TYPE:

(v i) ORIGINAL SOURCE:
(A) ORGANISM:
(B) STRAIN:
(C) INDIVIDUAL ISOLATE:
(D) DEVELOPMENTAL STAGE:
(E) HAPLOTYPE:
(F) TISSUE TYPE:
(G) CELL TYPE:
(H) CELL LINE:
(I) ORGANELLE:

(v i i) IMMEDIATE SOURCE:
(A) LIBRARY:
(B) CLONE:

(v i i i) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT:
(B) MAP POSITION:
(C) UNITS:

(i x) FEATURE:
(A) NAME/KEY:
(B) LOCATION:
(C) IDENTIFICATION METHOD:
(D) OTHER INFORMATION: /note= "sequence shown in Fig. 3 in 5'-3' direction"

(x) PUBLICATION INFORMATION:
(A) AUTHORS:
(B) TITLE:
(C) JOURNAL:
(D) VOLUME:
(E) ISSUE:
(F) PAGES:
(G) DATE:
(H) DOCUMENT NUMBER:
(I) FILING DATE:
(J) PUBLICATION DATE:
(K) RELEVANT RESIDUES IN SEQ ID NO:

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CACCTGACTC CTGAGGAGAA GTCTGCCGTT 30

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: other nucleic acid

(i i i) HYPOTHETICAL:

(i v) ANTI-SENSE:

(v) FRAGMENT TYPE:

(v i) ORIGINAL SOURCE:
(A) ORGANISM:
(B) STRAIN:

(C) INDIVIDUAL ISOLATE:
(D) DEVELOPMENTAL STAGE:
(E) HAPLOTYPE:
(F) TISSUE TYPE:
(G) CELL TYPE:
(H) CELL LINE:
(I) ORGANELLE:

(v i i) IMMEDIATE SOURCE:
(A) LIBRARY:
(B) CLONE:

(v i i i) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT:
(B) MAP POSITION:
(C) UNITS:

(i x) FEATURE:
(A) NAME/KEY:
(B) LOCATION:
(C) IDENTIFICATION METHOD:
(D) OTHER INFORMATION: /note= "sequence shown in Fig. 3 from 5'-3'direction"

(x) PUBLICATION INFORMATION:
(A) AUTHORS:
(B) TITLE:
(C) JOURNAL:
(D) VOLUME:
(E) ISSUE:
(F) PAGES:
(G) DATE:
(H) DOCUMENT NUMBER:
(I) FILING DATE:
(J) PUBLICATION DATE:
(K) RELEVANT RESIDUES IN SEQ ID NO:

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CACCTGACTC CTGTGGAGAA GTCTGCCGTT 30

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: other nucleic acid

(i i i) HYPOTHETICAL:

(i v) ANTI-SENSE:

(v) FRAGMENT TYPE:

(v i) ORIGINAL SOURCE:
(A) ORGANISM:
(B) STRAIN:
(C) INDIVIDUAL ISOLATE:
(D) DEVELOPMENTAL STAGE:
(E) HAPLOTYPE:
(F) TISSUE TYPE:
(G) CELL TYPE:
(H) CELL LINE:
(I) ORGANELLE:

(v i i) IMMEDIATE SOURCE:
(A) LIBRARY:
(B) CLONE:

(v i i i) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT:
(B) MAP POSITION:
(C) UNITS:

(i x) FEATURE:
(A) NAME/KEY:

(B) LOCATION:
(C) IDENTIFICATION METHOD:
(D) OTHER INFORMATION:

(x) PUBLICATION INFORMATION:
(A) AUTHORS:
(B) TITLE:
(C) JOURNAL:
(D) VOLUME:
(E) ISSUE:
(F) PAGES:
(G) DATE:
(H) DOCUMENT NUMBER:
(I) FILING DATE:
(J) PUBLICATION DATE:
(K) RELEVANT RESIDUES IN SEQ ID NO:

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:5:

ACACAACTGT GTGTTCACTA GC 22

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: other nucleic acid

(i i i) HYPOTHETICAL:

(i v) ANTI-SENSE:

(v) FRAGMENT TYPE:

(v i) ORIGINAL SOURCE:
(A) ORGANISM:
(B) STRAIN:
(C) INDIVIDUAL ISOLATE:
(D) DEVELOPMENTAL STAGE:
(E) HAPLOTYPE:
(F) TISSUE TYPE:
(G) CELL TYPE:
(H) CELL LINE:
(I) ORGANELLE:

(v i i) IMMEDIATE SOURCE:
(A) LIBRARY:
(B) CLONE:

(v i i i) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT:
(B) MAP POSITION:
(C) UNITS:

(i x) FEATURE:
(A) NAME/KEY:
(B) LOCATION:
(C) IDENTIFICATION METHOD:
(D) OTHER INFORMATION: /note="having biotin at the 5'end with a spacer"

(x) PUBLICATION INFORMATION:
(A) AUTHORS:
(B) TITLE:
(C) JOURNAL:
(D) VOLUME:
(E) ISSUE:
(F) PAGES:
(G) DATE:
(H) DOCUMENT NUMBER:
(I) FILING DATE:
(J) PUBLICATION DATE:
(K) RELEVANT RESIDUES IN SEQ ID NO:

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CAACTTCATC CACGTTCACC 20

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: other nucleic acid

(i i i) HYPOTHETICAL:

(i v) ANTI-SENSE:

(v) FRAGMENT TYPE:

(v i) ORIGINAL SOURCE:
(A) ORGANISM:
(B) STRAIN:
(C) INDIVIDUAL ISOLATE:
(D) DEVELOPMENTAL STAGE:
(E) HAPLOTYPE:
(F) TISSUE TYPE:
(G) CELL TYPE:
(H) CELL LINE:
(I) ORGANELLE:

(v i i) IMMEDIATE SOURCE:
(A) LIBRARY:
(B) CLONE:

(v i i i) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT:
(B) MAP POSITION:
(C) UNITS:

(i x) FEATURE:
(A) NAME/KEY:
(B) LOCATION:
(C) IDENTIFICATION METHOD:
(D) OTHER INFORMATION: /note= "sequence shown in Fig. 4 in 5'-3'direction"

(x) PUBLICATION INFORMATION:
(A) AUTHORS:
(B) TITLE:
(C) JOURNAL:
(D) VOLUME:
(E) ISSUE:
(F) PAGES:
(G) DATE:
(H) DOCUMENT NUMBER:
(I) FILING DATE:
(J) PUBLICATION DATE:
(K) RELEVANT RESIDUES IN SEQ ID NO:

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:7:

AGGTATGAGC AATTAAATGA CAGCTCA 27

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: other nucleic acid

(i i i) HYPOTHETICAL:

(i v) ANTI-SENSE:

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:
(A) ORGANISM:
(B) STRAIN:
(C) INDIVIDUAL ISOLATE:
(D) DEVELOPMENTAL STAGE:
(E) HAPLOTYPE:
(F) TISSUE TYPE:
(G) CELL TYPE:
(H) CELL LINE:
(I) ORGANELLE:

(vii) IMMEDIATE SOURCE:
(A) LIBRARY:
(B) CLONE:

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT:
(B) MAP POSITION:
(C) UNITS:

(ix) FEATURE:
(A) NAME/KEY:
(B) LOCATION:
(C) IDENTIFICATION METHOD:
(D) OTHER INFORMATION: /note="having biotin at the 5'end with a spacer"

(x) PUBLICATION INFORMATION:
(A) AUTHORS:
(B) TITLE:
(C) JOURNAL:
(D) VOLUME:
(E) ISSUE:
(F) PAGES:
(G) DATE:
(H) DOCUMENT NUMBER:
(I) FILING DATE:
(J) PUBLICATION DATE:
(K) RELEVANT RESIDUES IN SEQ ID NO:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GCAACCAGAG ACAACTGATC 20

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL:

(iv) ANTI-SENSE:

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:
(A) ORGANISM:
(B) STRAIN:
(C) INDIVIDUAL ISOLATE:
(D) DEVELOPMENTAL STAGE:
(E) HAPLOTYPE:
(F) TISSUE TYPE:
(G) CELL TYPE:
(H) CELL LINE:
(I) ORGANELLE:

(vii) IMMEDIATE SOURCE:
(A) LIBRARY:
(B) CLONE:

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT:
(B) MAP POSITION:
(C) UNITS:

(i x) FEATURE:
(A) NAME/KEY:
(B) LOCATION:
(C) IDENTIFICATION METHOD:
(D) OTHER INFORMATION:

(x) PUBLICATION INFORMATION:
(A) AUTHORS:
(B) TITLE:
(C) JOURNAL:
(D) VOLUME:
(E) ISSUE:
(F) PAGES:
(G) DATE:
(H) DOCUMENT NUMBER:
(I) FILING DATE:
(J) PUBLICATION DATE:
(K) RELEVANT RESIDUES IN SEQ ID NO:

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CGG TTG CTG GAA AGA TGC 18

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: other nucleic acid

(i i i) HYPOTHETICAL:

(i v) ANTI-SENSE:

(v) FRAGMENT TYPE:

(v i) ORIGINAL SOURCE:
(A) ORGANISM:
(B) STRAIN:
(C) INDIVIDUAL ISOLATE:
(D) DEVELOPMENTAL STAGE:
(E) HAPLOTYPE:
(F) TISSUE TYPE:
(G) CELL TYPE:
(H) CELL LINE:
(I) ORGANELLE:

(v i i) IMMEDIATE SOURCE:
(A) LIBRARY:
(B) CLONE:

(v i i i) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT:
(B) MAP POSITION:
(C) UNITS:

(i x) FEATURE:
(A) NAME/KEY:
(B) LOCATION:
(C) IDENTIFICATION METHOD:
(D) OTHER INFORMATION:

(x) PUBLICATION INFORMATION:
(A) AUTHORS:
(B) TITLE:
(C) JOURNAL:
(D) VOLUME:
(E) ISSUE:
(F) PAGES:
(G) DATE:
(H) DOCUMENT NUMBER:
(I) FILING DATE:
(J) PUBLICATION DATE:
(K) RELEVANT RESIDUES IN SEQ ID NO:

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CAG CAG GAT AAG TAT GAG 18

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: other nucleic acid

(i i i) HYPOTHETICAL:

(i v) ANTI-SENSE:

(v) FRAGMENT TYPE:

(v i) ORIGINAL SOURCE:
(A) ORGANISM:
(B) STRAIN:
(C) INDIVIDUAL ISOLATE:
(D) DEVELOPMENTAL STAGE:
(E) HAPLOTYPE:
(F) TISSUE TYPE:
(G) CELL TYPE:
(H) CELL LINE:
(I) ORGANELLE:

(v i i) IMMEDIATE SOURCE:
(A) LIBRARY:
(B) CLONE:

(v i i i) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT:
(B) MAP POSITION:
(C) UNITS:

(i x) FEATURE:
(A) NAME/KEY:
(B) LOCATION:
(C) IDENTIFICATION METHOD:
(D) OTHER INFORMATION:

(x) PUBLICATION INFORMATION:
(A) AUTHORS:
(B) TITLE:
(C) JOURNAL:
(D) VOLUME:
(E) ISSUE:
(F) PAGES:
(G) DATE:
(H) DOCUMENT NUMBER:
(I) FILING DATE:
(J) PUBLICATION DATE:
(K) RELEVANT RESIDUES IN SEQ ID NO:

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GGC CGG GTG GAC AAC TAC 18

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: other nucleic acid

(i i i) HYPOTHETICAL:

(i v) ANTI-SENSE:

(v) FRAGMENT TYPE:

-continued

(v i) ORIGINAL SOURCE:
(A) ORGANISM:
(B) STRAIN:
(C) INDIVIDUAL ISOLATE:
(D) DEVELOPMENTAL STAGE:
(E) HAPLOTYPE:
(F) TISSUE TYPE:
(G) CELL TYPE:
(H) CELL LINE:
(I) ORGANELLE:

(v i i) IMMEDIATE SOURCE:
(A) LIBRARY:
(B) CLONE:

(v i i i) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT:
(B) MAP POSITION:
(C) UNITS:

(i x) FEATURE:
(A) NAME/KEY:
(B) LOCATION:
(C) IDENTIFICATION METHOD:
(D) OTHER INFORMATION:

(x) PUBLICATION INFORMATION:
(A) AUTHORS:
(B) TITLE:
(C) JOURNAL:
(D) VOLUME:
(E) ISSUE:
(F) PAGES:
(G) DATE:
(H) DOCUMENT NUMBER:
(I) FILING DATE:
(J) PUBLICATION DATE:
(K) RELEVANT RESIDUES IN SEQ ID NO:

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:12:

TTG GAG CAG GTT AAA CAT 18

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: other nucleic acid

(i i i) HYPOTHETICAL:

(i v) ANTI-SENSE:

(v) FRAGMENT TYPE:

(v i) ORIGINAL SOURCE:
(A) ORGANISM:
(B) STRAIN:
(C) INDIVIDUAL ISOLATE:
(D) DEVELOPMENTAL STAGE:
(E) HAPLOTYPE:
(F) TISSUE TYPE:
(G) CELL TYPE:
(H) CELL LINE:
(I) ORGANELLE:

(v i i) IMMEDIATE SOURCE:
(A) LIBRARY:
(B) CLONE:

(v i i i) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT:
(B) MAP POSITION:
(C) UNITS:

(i x) FEATURE:
(A) NAME/KEY:
(B) LOCATION:
(C) IDENTIFICATION METHOD:
(D) OTHER INFORMATION:

(x) PUBLICATION INFORMATION:
(A) AUTHORS:
(B) TITLE:
(C) JOURNAL:
(D) VOLUME:
(E) ISSUE:
(F) PAGES:
(G) DATE:
(H) DOCUMENT NUMBER:
(I) FILING DATE:
(J) PUBLICATION DATE:
(K) RELEVANT RESIDUES IN SEQ ID NO:

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:13:

T GAT GAG GAG TAC TGG AA 18

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: other nucleic acid

(i i i) HYPOTHETICAL:

(i v) ANTI-SENSE:

(v) FRAGMENT TYPE:

(v i) ORIGINAL SOURCE:
(A) ORGANISM:
(B) STRAIN:
(C) INDIVIDUAL ISOLATE:
(D) DEVELOPMENTAL STAGE:
(E) HAPLOTYPE:
(F) TISSUE TYPE:
(G) CELL TYPE:
(H) CELL LINE:
(I) ORGANELLE:

(v i i) IMMEDIATE SOURCE:
(A) LIBRARY:
(B) CLONE:

(v i i i) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT:
(B) MAP POSITION:
(C) UNITS:

(i x) FEATURE:
(A) NAME/KEY:
(B) LOCATION:
(C) IDENTIFICATION METHOD:
(D) OTHER INFORMATION:

(x) PUBLICATION INFORMATION:
(A) AUTHORS:
(B) TITLE:
(C) JOURNAL:
(D) VOLUME:
(E) ISSUE:
(F) PAGES:
(G) DATE:
(H) DOCUMENT NUMBER:
(I) FILING DATE:
(J) PUBLICATION DATE:
(K) RELEVANT RESIDUES IN SEQ ID NO:

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GG TTA CTG GAG AGA CAC T 18

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: other nucleic acid

(i i i) HYPOTHETICAL:

(i v) ANTI-SENSE:

(v) FRAGMENT TYPE:

(v i) ORIGINAL SOURCE:
(A) ORGANISM:
(B) STRAIN:
(C) INDIVIDUAL ISOLATE:
(D) DEVELOPMENTAL STAGE:
(E) HAPLOTYPE:
(F) TISSUE TYPE:
(G) CELL TYPE:
(H) CELL LINE:
(I) ORGANELLE:

(v i i) IMMEDIATE SOURCE:
(A) LIBRARY:
(B) CLONE:

(v i i i) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT:
(B) MAP POSITION:
(C) UNITS:

(i x) FEATURE:
(A) NAME/KEY:
(B) LOCATION:
(C) IDENTIFICATION METHOD:
(D) OTHER INFORMATION:

(x) PUBLICATION INFORMATION:
(A) AUTHORS:
(B) TITLE:
(C) JOURNAL:
(D) VOLUME:
(E) ISSUE:
(F) PAGES:
(G) DATE:
(H) DOCUMENT NUMBER:
(I) FILING DATE:
(J) PUBLICATION DATE:
(K) RELEVANT RESIDUES IN SEQ ID NO:

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:15:

TTC TTG GAG TAC TCT ACG 18

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: other nucleic acid

(i i i) HYPOTHETICAL:

(i v) ANTI-SENSE:

(v) FRAGMENT TYPE:

(v i) ORIGINAL SOURCE:
(A) ORGANISM:
(B) STRAIN:
(C) INDIVIDUAL ISOLATE:
(D) DEVELOPMENTAL STAGE:
(E) HAPLOTYPE:
(F) TISSUE TYPE:
(G) CELL TYPE:
(H) CELL LINE:
(I) ORGANELLE:

(v i i) IMMEDIATE SOURCE:
(A) LIBRARY:
(B) CLONE:

(v i i i) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT:
(B) MAP POSITION:
(C) UNITS:

(i x) FEATURE:
(A) NAME/KEY:
(B) LOCATION:
(C) IDENTIFICATION METHOD:
(D) OTHER INFORMATION:

(x) PUBLICATION INFORMATION:
(A) AUTHORS:
(B) TITLE:
(C) JOURNAL:
(D) VOLUME:
(E) ISSUE:
(F) PAGES:
(G) DATE:
(H) DOCUMENT NUMBER:
(I) FILING DATE:
(J) PUBLICATION DATE:
(K) RELEVANT RESIDUES IN SEQ ID NO:

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:16:

TGG CAG GGT AAG TAT AAG 18

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: other nucleic acid

(i i i) HYPOTHETICAL:

(i v) ANTI-SENSE:

(v) FRAGMENT TYPE:

(v i) ORIGINAL SOURCE:
(A) ORGANISM:
(B) STRAIN:
(C) INDIVIDUAL ISOLATE:
(D) DEVELOPMENTAL STAGE:
(E) HAPLOTYPE:
(F) TISSUE TYPE:
(G) CELL TYPE:
(H) CELL LINE:
(I) ORGANELLE:

(v i i) IMMEDIATE SOURCE:
(A) LIBRARY:
(B) CLONE:

(v i i i) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT:
(B) MAP POSITION:
(C) UNITS:

(i x) FEATURE:
(A) NAME/KEY:
(B) LOCATION:
(C) IDENTIFICATION METHOD:
(D) OTHER INFORMATION:

(x) PUBLICATION INFORMATION:
(A) AUTHORS:
(B) TITLE:
(C) JOURNAL:
(D) VOLUME:
(E) ISSUE:
(F) PAGES:
(G) DATE:
(H) DOCUMENT NUMBER:
(I) FILING DATE:
(J) PUBLICATION DATE:
(K) RELEVANT RESIDUES IN SEQ ID NO:

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:17:

A GAC AGG CGG GCC CTG GT 18

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: other nucleic acid

(i i i) HYPOTHETICAL:

(i v) ANTI-SENSE:

(v) FRAGMENT TYPE:

(v i) ORIGINAL SOURCE:
(A) ORGANISM:
(B) STRAIN:
(C) INDIVIDUAL ISOLATE:
(D) DEVELOPMENTAL STAGE:
(E) HAPLOTYPE:
(F) TISSUE TYPE:
(G) CELL TYPE:
(H) CELL LINE:
(I) ORGANELLE:

(v i i) IMMEDIATE SOURCE:
(A) LIBRARY:
(B) CLONE:

(v i i i) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT:
(B) MAP POSITION:
(C) UNITS:

(i x) FEATURE:
(A) NAME/KEY:
(B) LOCATION:
(C) IDENTIFICATION METHOD:
(D) OTHER INFORMATION:

(x) PUBLICATION INFORMATION:
(A) AUTHORS:
(B) TITLE:
(C) JOURNAL:
(D) VOLUME:
(E) ISSUE:
(F) PAGES:
(G) DATE:
(H) DOCUMENT NUMBER:
(I) FILING DATE:
(J) PUBLICATION DATE:
(K) RELEVANT RESIDUES IN SEQ ID NO:

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:18:

G TAT CTG CAC AGA GGC AT 18

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: other nucleic acid

(i i i) HYPOTHETICAL:

(i v) ANTI-SENSE:

(v) FRAGMENT TYPE:

(v i) ORIGINAL SOURCE:
(A) ORGANISM:
(B) STRAIN:
(C) INDIVIDUAL ISOLATE:
(D) DEVELOPMENTAL STAGE:
(E) HAPLOTYPE:
(F) TISSUE TYPE:
(G) CELL TYPE:
(H) CELL LINE:
(I) ORGANELLE:

(v i i) IMMEDIATE SOURCE:
(A) LIBRARY:
(B) CLONE:

(v i i i) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT:
(B) MAP POSITION:
(C) UNITS:

(i x) FEATURE:
(A) NAME/KEY:
(B) LOCATION:
(C) IDENTIFICATION METHOD:
(D) OTHER INFORMATION:

(x) PUBLICATION INFORMATION:
(A) AUTHORS:
(B) TITLE:
(C) JOURNAL:
(D) VOLUME:
(E) ISSUE:
(F) PAGES:
(G) DATE:
(H) DOCUMENT NUMBER:
(I) FILING DATE:
(J) PUBLICATION DATE:
(K) RELEVANT RESIDUES IN SEQ ID NO:

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:19:

TTG AAG CAG GAT AAG TTT 18

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: other nucleic acid

(i i i) HYPOTHETICAL:

(i v) ANTI-SENSE:

(v) FRAGMENT TYPE:

(v i) ORIGINAL SOURCE:
(A) ORGANISM:
(B) STRAIN:
(C) INDIVIDUAL ISOLATE:
(D) DEVELOPMENTAL STAGE:
(E) HAPLOTYPE:
(F) TISSUE TYPE:
(G) CELL TYPE:
(H) CELL LINE:
(I) ORGANELLE:

(v i i) IMMEDIATE SOURCE:
(A) LIBRARY:
(B) CLONE:

(v i i i) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT:
(B) MAP POSITION:
(C) UNITS:

(i x) FEATURE:
(A) NAME/KEY:
(B) LOCATION:
(C) IDENTIFICATION METHOD:
(D) OTHER INFORMATION:

(x) PUBLICATION INFORMATION:
(A) AUTHORS:
(B) TITLE:
(C) JOURNAL:
(D) VOLUME:
(E) ISSUE:
(F) PAGES:
(G) DATE:
(H) DOCUMENT NUMBER:
(I) FILING DATE:
(J) PUBLICATION DATE:
(K) RELEVANT RESIDUES IN SEQ ID NO:

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:20:

TGC AGA CAC AAC TAC GGG 18

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: other nucleic acid

(i i i) HYPOTHETICAL:

(i v) ANTI-SENSE:

(v) FRAGMENT TYPE:

(v i) ORIGINAL SOURCE:
(A) ORGANISM:
(B) STRAIN:
(C) INDIVIDUAL ISOLATE:
(D) DEVELOPMENTAL STAGE:
(E) HAPLOTYPE:
(F) TISSUE TYPE:
(G) CELL TYPE:
(H) CELL LINE:
(I) ORGANELLE:

(v i i) IMMEDIATE SOURCE:
(A) LIBRARY:
(B) CLONE:

(v i i i) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT:
(B) MAP POSITION:
(C) UNITS:

(i x) FEATURE:
(A) NAME/KEY:
(B) LOCATION:
(C) IDENTIFICATION METHOD:
(D) OTHER INFORMATION: /note="having biotin at the 5'end with a spacer"

(x) PUBLICATION INFORMATION:
(A) AUTHORS:
(B) TITLE:
(C) JOURNAL:
(D) VOLUME:
(E) ISSUE:
(F) PAGES:
(G) DATE:
(H) DOCUMENT NUMBER:
(I) FILING DATE:
(J) PUBLICATION DATE:
(K) RELEVANT RESIDUES IN SEQ ID NO:

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:21:

TTCTTCAATG GGACGGAGCG 20

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: other nucleic acid

(i i i) HYPOTHETICAL:

(i v) ANTI-SENSE:

(v) FRAGMENT TYPE:

(v i) ORIGINAL SOURCE:
(A) ORGANISM:
(B) STRAIN:
(C) INDIVIDUAL ISOLATE:
(D) DEVELOPMENTAL STAGE:
(E) HAPLOTYPE:
(F) TISSUE TYPE:
(G) CELL TYPE:
(H) CELL LINE:
(I) ORGANELLE:

(v i i) IMMEDIATE SOURCE:
(A) LIBRARY:
(B) CLONE:

(v i i i) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT:
(B) MAP POSITION:
(C) UNITS:

(i x) FEATURE:
(A) NAME/KEY:
(B) LOCATION:
(C) IDENTIFICATION METHOD:
(D) OTHER INFORMATION: /note="having biotin at the 5'end with a spacer"

(x) PUBLICATION INFORMATION:
(A) AUTHORS:
(B) TITLE:
(C) JOURNAL:
(D) VOLUME:
(E) ISSUE:
(F) PAGES:
(G) DATE:
(H) DOCUMENT NUMBER:
(I) FILING DATE:
(J) PUBLICATION DATE:

(K) RELEVANT RESIDUES IN SEQ ID NO:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

GCCGCTGCAC TGTGAAGCTC TC 22

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL:

(iv) ANTI-SENSE:

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:
(A) ORGANISM:
(B) STRAIN:
(C) INDIVIDUAL ISOLATE:
(D) DEVELOPMENTAL STAGE:
(E) HAPLOTYPE:
(F) TISSUE TYPE:
(G) CELL TYPE:
(H) CELL LINE:
(I) ORGANELLE:

(vii) IMMEDIATE SOURCE:
(A) LIBRARY:
(B) CLONE:

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT:
(B) MAP POSITION:
(C) UNITS:

(ix) FEATURE:
(A) NAME/KEY:
(B) LOCATION:
(C) IDENTIFICATION METHOD:
(D) OTHER INFORMATION:

(x) PUBLICATION INFORMATION:
(A) AUTHORS:
(B) TITLE:
(C) JOURNAL:
(D) VOLUME:
(E) ISSUE:
(F) PAGES:
(G) DATE:
(H) DOCUMENT NUMBER:
(I) FILING DATE:
(J) PUBLICATION DATE:
(K) RELEVANT RESIDUES IN SEQ ID NO:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

CAGCAGGATA AGTA 14

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL:

(iv) ANTI-SENSE:

(v) FRAGMENT TYPE:

(v i) ORIGINAL SOURCE:
(A) ORGANISM:
(B) STRAIN:
(C) INDIVIDUAL ISOLATE:
(D) DEVELOPMENTAL STAGE:
(E) HAPLOTYPE:
(F) TISSUE TYPE:
(G) CELL TYPE:
(H) CELL LINE:
(I) ORGANELLE:

(v i i) IMMEDIATE SOURCE:
(A) LIBRARY:
(B) CLONE:

(v i i i) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT:
(B) MAP POSITION:
(C) UNITS:

(i x) FEATURE:
(A) NAME/KEY:
(B) LOCATION:
(C) IDENTIFICATION METHOD:
(D) OTHER INFORMATION:

(x) PUBLICATION INFORMATION:
(A) AUTHORS:
(B) TITLE:
(C) JOURNAL:
(D) VOLUME:
(E) ISSUE:
(F) PAGES:
(G) DATE:
(H) DOCUMENT NUMBER:
(I) FILING DATE:
(J) PUBLICATION DATE:
(K) RELEVANT RESIDUES IN SEQ ID NO:

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:24:

GGTTACTGGA GAG 13

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: other nucleic acid

(i i i) HYPOTHETICAL:

(i v) ANTI-SENSE:

(v) FRAGMENT TYPE:

(v i) ORIGINAL SOURCE:
(A) ORGANISM:
(B) STRAIN:
(C) INDIVIDUAL ISOLATE:
(D) DEVELOPMENTAL STAGE:
(E) HAPLOTYPE:
(F) TISSUE TYPE:
(G) CELL TYPE:
(H) CELL LINE:
(I) ORGANELLE:

(v i i) IMMEDIATE SOURCE:
(A) LIBRARY:
(B) CLONE:

(v i i i) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT:

(B) MAP POSITION:
(C) UNITS:

(i x) FEATURE:
(A) NAME/KEY:
(B) LOCATION:
(C) IDENTIFICATION METHOD:
(D) OTHER INFORMATION:

(x) PUBLICATION INFORMATION:
(A) AUTHORS:
(B) TITLE:
(C) JOURNAL:
(D) VOLUME:
(E) ISSUE:
(F) PAGES:
(G) DATE:
(H) DOCUMENT NUMBER:
(I) FILING DATE:
(J) PUBLICATION DATE:
(K) RELEVANT RESIDUES IN SEQ ID NO:

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:25:

GTATCTGCAC AGAGG 15

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 90 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: other nucleic acid

(i i i) HYPOTHETICAL:

(i v) ANTI-SENSE:

(v) FRAGMENT TYPE:

(v i) ORIGINAL SOURCE:
(A) ORGANISM:
(B) STRAIN:
(C) INDIVIDUAL ISOLATE:
(D) DEVELOPMENTAL STAGE:
(E) HAPLOTYPE:
(F) TISSUE TYPE:
(G) CELL TYPE:
(H) CELL LINE:
(I) ORGANELLE:

(v i i) IMMEDIATE SOURCE:
(A) LIBRARY:
(B) CLONE:

(v i i i) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT:
(B) MAP POSITION:
(C) UNITS:

(i x) FEATURE:
(A) NAME/KEY:
(B) LOCATION:
(C) IDENTIFICATION METHOD:
(D) OTHER INFORMATION: /note= "DRB5*0101"

(x) PUBLICATION INFORMATION:
(A) AUTHORS:
(B) TITLE:
(C) JOURNAL:
(D) VOLUME:
(E) ISSUE:
(F) PAGES:
(G) DATE:
(H) DOCUMENT NUMBER:
(I) FILING DATE:
(J) PUBLICATION DATE:

(K) RELEVANT RESIDUES IN SEQ ID NO:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

GGGGACACCC GACCACGTTT CTTGCAGCAG GATAAGTATG AGTGTCATTT 50

CTTCAACGGG ACGGAGCGGG TGCGGTTCCT GCACAGAGAC 90

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 90 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL:

(iv) ANTI-SENSE:

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:
(A) ORGANISM:
(B) STRAIN:
(C) INDIVIDUAL ISOLATE:
(D) DEVELOPMENTAL STAGE:
(E) HAPLOTYPE:
(F) TISSUE TYPE:
(G) CELL TYPE:
(H) CELL LINE:
(I) ORGANELLE:

(vii) IMMEDIATE SOURCE:
(A) LIBRARY:
(B) CLONE:

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT:
(B) MAP POSITION:
(C) UNITS:

(ix) FEATURE:
(A) NAME/KEY:
(B) LOCATION:
(C) IDENTIFICATION METHOD:
(D) OTHER INFORMATION: /note= "DRB1*1201"

(x) PUBLICATION INFORMATION:
(A) AUTHORS:
(B) TITLE:
(C) JOURNAL:
(D) VOLUME:
(E) ISSUE:
(F) PAGES:
(G) DATE:
(H) DOCUMENT NUMBER:
(I) FILING DATE:
(J) PUBLICATION DATE:
(K) RELEVANT RESIDUES IN SEQ ID NO:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

GGGGACACCA GACCACGTTT CTTGGAGTAC TCTACGGGTG AGTGTTATTT 50

CTTCAATGGG ACGGAGCGGG TGCGGTTACT GGAGAGACAC 90

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 90 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(i i i) HYPOTHETICAL:

(i v) ANTI-SENSE:

(v) FRAGMENT TYPE:

(v i) ORIGINAL SOURCE:
(A) ORGANISM:
(B) STRAIN:
(C) INDIVIDUAL ISOLATE:
(D) DEVELOPMENTAL STAGE:
(E) HAPLOTYPE:
(F) TISSUE TYPE:
(G) CELL TYPE:
(H) CELL LINE:
(I) ORGANELLE:

(v i i) IMMEDIATE SOURCE:
(A) LIBRARY:
(B) CLONE:

(v i i i) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT:
(B) MAP POSITION:
(C) UNITS:

(i x) FEATURE:
(A) NAME/KEY:
(B) LOCATION:
(C) IDENTIFICATION METHOD:
(D) OTHER INFORMATION: /note= "DRB1*0901"

(x) PUBLICATION INFORMATION:
(A) AUTHORS:
(B) TITLE:
(C) JOURNAL:
(D) VOLUME:
(E) ISSUE:
(F) PAGES:
(G) DATE:
(H) DOCUMENT NUMBER:
(I) FILING DATE:
(J) PUBLICATION DATE:
(K) RELEVANT RESIDUES IN SEQ ID NO:

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:28:

NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN 50

NTTCAACGGG ACGGAGCGGG TGCGGTATCT GCACAGAGGC 90

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 90 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: other nucleic acid

(i i i) HYPOTHETICAL:

(i v) ANTI-SENSE:

(v) FRAGMENT TYPE:

(v i) ORIGINAL SOURCE:
(A) ORGANISM:
(B) STRAIN:
(C) INDIVIDUAL ISOLATE:
(D) DEVELOPMENTAL STAGE:
(E) HAPLOTYPE:
(F) TISSUE TYPE:
(G) CELL TYPE:
(H) CELL LINE:
(I) ORGANELLE:

-continued

(v i i) IMMEDIATE SOURCE:
(A) LIBRARY:
(B) CLONE:

(v i i i) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT:
(B) MAP POSITION:
(C) UNITS:

(i x) FEATURE:
(A) NAME/KEY:
(B) LOCATION:
(C) IDENTIFICATION METHOD:
(D) OTHER INFORMATION: /note= "DRB1*0101"

(x) PUBLICATION INFORMATION:
(A) AUTHORS:
(B) TITLE:
(C) JOURNAL:
(D) VOLUME:
(E) ISSUE:
(F) PAGES:
(G) DATE:
(H) DOCUMENT NUMBER:
(I) FILING DATE:
(J) PUBLICATION DATE:
(K) RELEVANT RESIDUES IN SEQ ID NO:

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:29:

GGGGACACCC GACCACGTTT CTTGTGGCAG CTTAAGTTTG AATGTCATTT 50

CTTCAATGGG ACGGAGCGGG TGCGGTTGCT GGAAAGATGC 90

What is claimed is:

1. A process for immobilizing an HLA-DRB oligonucleotide sequence in a microtiter well, comprising the steps of:

preparing a base sequence in which HLA-DRB oligonucleotide sequences specifically hybridizable with a target nucleic acid are repeated in tandem 5 to 200 times,

inserting the base sequence into a vector which is capable of producing a single stranded nucleic acid to prepare a recombinant vector,

transforming a microorganism with the recombinant vector, and culturing the microorganism to amplify the number of the base sequence,

isolating the amplified base sequence in which the HLA-DRB oligonucleotide sequences specifically hybridizable with the target nucleic acid are repeated in tandem 5 to 200 times, and

immobilizing the amplified base sequence in the microtiter well by irradiating the microtiter well in the presence of the amplified base sequence with ultraviolet light.

2. The process according to claim 1, wherein the vector is a phage or a phage-plasmid.

3. A microtiter well which is produced by the process of claim 1.

4. A microtiter well which is produced by the process of claim 2.

* * * * *

Detailed Description Text - DETX (24):

When the label is directly detectable, that is, when the label is, for example, a radioisotope, a fluorescent substance or a dye, the detection procedure may be conducted in such a state that a labelled nucleic acid is bonded to a solid phase, or alternatively the detection procedure may be conducted by liberating the label into a solution in such a state that it is bonded to the nucleic acid or released from the nucleic acid and then conducting the detection according to the label. On the other hand, when the label is indirectly detectable, that is, when the label is a ligand capable of causing a specific binding reaction, such as biotin or hapten, the detection procedure can be conducted by a method commonly used in the detection of this type, that is, by use of an acceptor (for example, avidin or antibody) to which a label capable of directly generating a signal or an enzyme catalyzing a signal generating reaction has been bound. The acceptor may be previously added in the hybridization procedure. In this case, the binding process of the ligand to the acceptor can be conducted simultaneously with the hybridization step with the result that the whole process can be simplified.

Detailed Description Text - DETX (12):

It was surprising that the single stranded nucleic acids derived from the phages or the composite vectors comprising a phage and a plasmid which contain a gene to be detected can be directly immobilized on a microtiter well through a nonspecific absorption efficiently. In particular, the single stranded nucleic acids having 1 k bs to 8 k bs size can be directly immobilized on a microtiter well in a large amount. Thus, it is preferred to use the single stranded nucleic acids having 1 k bs to 8 k bs size. In addition, a microtiter well on which the single stranded nucleic acids having 1 k bs to 8 k bs size are immobilized ensures a detection of a target nucleic acid in a high sensitivity. The adsorption of the single stranded nucleic acid on the microtiter well can be preferably enhanced by irradiation of ultraviolet light or by adding MgCl.sub.2 (Japanese Patent Laid-Open Publication No. 219400/1986).

```
     (FILE 'HOME' ENTERED AT 16:23:06 ON 31 MAY 2003)

     FILE 'MEDLINE, CAPLUS, BIOSIS, EMBASE, SCISEARCH' ENTERED AT 16:23:16 ON
     31 MAY 2003
L1          126 S DNA (P)(UV OR ULTRAVIOLET OR DAMAGE OR DAMAGED OR
NICKED)(P)(
L2           54 DUPLICATE REMOVE L1 (72 DUPLICATES REMOVED)
L3           46 S L2 AND PY<2001
L4         3150 S DNA-PK?
L5            0 S DNA-PK(P)MICROTITER
L6            4 S ANTI-DNA-PK
L7            1 S L4 AND MICROTITER
```

US005919626A

United States Patent [19]

Shi et al.

[11] **Patent Number: 5,919,626**

[45] **Date of Patent: Jul. 6, 1999**

[54] **ATTACHMENT OF UNMODIFIED NUCLEIC ACIDS TO SILANIZED SOLID PHASE SURFACES**

[75] Inventors: **Jufang Shi**, Baltimore; **Michael T. Boyce-Jacino**, Finksburg, both of Md.

[73] Assignee: **Orchid Bio Computer, Inc.**, Baltimore, Md.

[21] Appl. No.: **08/870,010**

[22] Filed: **Jun. 6, 1997**

[51] **Int. Cl.**[6] **C12Q 1/68**; G01N 33/00; C12M 1/00; C07H 21/02

[52] **U.S. Cl.** **435/6**; 435/91.1; 435/287.2; 436/94; 536/23.1; 536/24.3; 536/25.3

[58] **Field of Search** 435/6, 91.1, 91.2, 435/183, 287.2; 536/23.1, 24.3, 25.3; 935/76, 77, 86, 87; 436/94

[56] **References Cited**

U.S. PATENT DOCUMENTS

4,521,509	6/1985	Benkovic et al.	435/6
4,656,127	4/1987	Mundy	435/6
5,075,217	12/1991	Weber	435/6
5,175,082	12/1992	Jeffreys	435/6
5,302,509	4/1994	Cheeseman	435/6
5,518,900	5/1996	Nikiforov et al.	435/91.1

FOREIGN PATENT DOCUMENTS

2650840	8/1989	France .
WO 90/11369	10/1990	WIPO .
WO 90/13668	11/1990	WIPO .
WO 91/14003	9/1991	WIPO .
WO 92/15712	9/1992	WIPO .
WO 95/17676	6/1995	WIPO .

OTHER PUBLICATIONS

Blanchard, A.P., "High Density Oligonucleotide Arrays," *Biosensors & Bioelectronics* 11 (6/7) 687–690 (1996).

Southern, E.M., "Detection of Specific Sequences Among DNA Fragments Separated By Gel Electrophoresis," *J. Mol. Biol.* 98 503–517 (1975).

Pl Nyrén, et al., Solid Phase DNA Minisequencing by an Enzymatic Luminometric Inorganic Phyrophosate Detection Assay, (1993) , Analytical Biochemistry 208, 171–175.

Ann–Christine Syvänen, et al. Identification of Individuals by Analysis of Biallelic DNA Markers, Using PCR and Solid–Phase Minisequencing, (1993) Am. J. Hum, Genet. 52:46–59.

Mohan N. Kuppuswamy, et al., Single nucleotide primer extension to detect genetic diseases: Experimental application to hemophilia B (factor IX) and cystic fibrosis genes, (Feb. 1991). Proc. Natl. Acad. Sci. USA vol. 88, pp. 1143–1147.

Deborah A. Nickerson, et al., Automated DNA diagnostics using an ELISA–based oligonucleotide ligation assay, (Nov. 1990) Proc. Natl. Acad. Sci. USA vol. 87, pp. 8923–8927.

Ulf Landegren, A Ligase–Mediated Gene Detection Technique, (Aug. 26, 1988), Sci. vol. 241, pp. 1077–1080.

Arthur Kornberg, et al., Deoxyribonucleases, DNA Replication, Second Edition (1992).

J. Hillel, et al. DNA Fingerprints Applied to Gene Introgression in Breeding Programs (Mar. 1990), Genetics 124: 783–789.

J. Hillel, et al., DNA Fingerprints of poultry, (1989), Animan Genetics, 20, 145–155.

Russell G. Higuchi, et al., Production of single–stranded DNA templates by exonuclease digestion following the polymerase chain reaction, (Nov. 14, 1989, vol. 17.

Ulf B. Gyllensten, et al., Generation of single–stranded DNA by the polymerase chain reaction and its application to direct sequencing of the HLA–DQA locus, (Oct. 1988) Proc. Natl. Acad. Sci. USA vol. 85, pp. 7652–7656.

Mirta Mihovilovic, et al., Biotechniques, (1989) vol. 7, No. 1.

Uwe Maskos, et al., Oligonucleotide hybridisations on glass supports: a novel linker for oligonucleotidsynthesis and hybridisation properties of oligonucleotides synthesised in situ, (1992) Nucleic Acids Research, vol. 20, No. 7 1679–1684.

Ann–Christine Syvänen, A Primer–Guided Nucleotide Incorporation Assay in the Genotyping of Apolipoprotein E, (1990) Genomics 8, 684–692.

Søren Richard Rasmussen, et al., Covalent Immobilization of DNA onto Polystyrene Microwells: The Molecules Are only Bound at the 5'End (1991) Analytical Biochemistry 198, 138–142.

Mark X. Sliwkowski, et al., Resolution of sulphydryl oxidase from γ–glutamyltransferase in bovine milk by alent chromatography on cysteinylsuccinamidoproply–glass, (1991) Biochem. J. 209, 731–739. vol. 209.

Eoin Fahy, et al., Design and synthesis of polyacrylamide–based oligonucleotide supports for use in nucleic acid diagnostics, (1993) Nucleic Acids Research, vol. 21, No. 8 1819–1826.

A.J. Jeffreys, et al., DNA fingerprints of dogs and cats (1987) Animal Genetics, 18, 1–15.

L. Jones, Identical twin marrow transplantation for 5 patients with chronic myeloid leukaemia: Role of DNA finger–printing to confirm monozygosity in 3 cases, (1987) Eur J. Haematol 39:144–147.

S.S. Moore, et al., The Conversation of Dinucleotide Microsatellites among Mammalian Genomes Allow Use of Heterologous PCR Primer Pairs in Closely Related Species (1991) Genomics 10, 654–660–.

(List continued on next page.)

Primary Examiner—Bradley L. Sisson

Attorney, Agent, or Firm—Howrey & Simon; Jeffrey Auerbach; Kevin McCabe

[57] **ABSTRACT**

The invention relates to a simple, cost effective method for immobilizing synthetic nucleic acid molecules onto a solid support. The invention further concerns the use of such immobilized molecules in nucleic acid hybridization assays, sequencing by hybridization assays, and genetic analyses and combinatorial analyses involving nucleic acids or proteins for screening applications.

29 Claims, No Drawings

1026 9 1-3, 5, ~~7~~, 12-15, 17

OTHER PUBLICATIONS

Ian C. Gray, et al., Evolutionary transience of hypervariable minisatellites in man and the primates (1991) Proc. R. Soc. Lond. B. 243 241–253.
A. J. Jeffreys, et al., Individual–specific 'fingerprints' of human DNA (Jul. 4, 1985) Nature vol. 316.
Alec J. Jeffreys, et al., DNA "Fingerprints" and Segregation Analysis of Multiple Markers in Human Pedigrees (1986) *Am J Hum Genet* 39:11–24.
Theo T. Nikiforov, et al., The Use of Phosphorothioate Primers and Exonuclease Hydrolysis for the Preperation of Single–stranded PCR Products and their Detection by Solid–phase Hybridization (1994) 3:285–291.
M.H. Skolnick and et al., Strategies for detecting and characterizing restriction fragment length polymorphisms (RFLP's) (1982) Cytogenet. Cell Genet. 32: 58–67.
John A.L. Armour, et al., Recent advances in minisatellite biology, (Jul. 1992) vol. 307, No. 1, 113–115.
J. F. Gusella, DNA Polymorphism and Human, (1986) Ann. Rev. Bichem. 55:831–54.
Theo T. Nikiforov, et al., Genetic Bit Analysis: a solid phase method for typing single nucleotide polymorphisms (1994) Nucleic Acids Research, vol. 22, No. 20 4167–4175.
Patrik Dahlén, et al., Sensitive detection of genes by sandwich hybridization and time–resolved fluorometry, (1987) Molecular and Cellular Probes 1, 159–168.
N.D. Sinha, et al., Polymer support oligonucleotide synthesis XVIII: use of β–cyanoethyl–N–dialkylamino–/N–morpholino phosphoramidite of deoxynucleosides for the synthesis of DNA fragments simplifying deprotection and isolation of the final product (1984) vol. 12 No. 11.
E.M. Southern, et al., Arrays of complementary oligonucleotides for analysing the hybridisation behaviour of nucleic acids (1994) Nucleic Acids Research, vol. 22, No. 8, 1368–1373.
Ann Caviani Pease, et al., Light–generated oligonucleotide arrays for rapid DNA sequence analysis, (May 1994) Proc. Natl. Acad. Sci. USA vol. 91, pp. 5022–5026.
Zhen Guo, et al., Direct fluorescence analysis of genetic polymorphisms by hybridization with oligonucleotide arrays on glass supports (1994) Nucleic Acids Research, vol. 22, No. 24 pp. 5456–5465.
Johann Ott, et al., Protection of Oligonucleotide Primers against Degradation by DNA Polymerase I, (1987) Bochemistry, 26, 8237–8241.
Fritz Eckstein, et al., Synthesis and Properties of Diastereoisomers of Adenosine 5'–(*O*–1–Thiotriphosphate) and Adenosine 5'–(*O*2 –Thiotriphosphate), (1976) Biochemistry, vol. 15, No. 8.
K.R. Khrapko, et al., A method of DNA sequencing by hybridization with oligonucleotide matrix, (1992) DNA Sequence vol. 1, pp. 375–388.
Stanley Tabor, et al., Effect of maganese ions on the incorporation of dideoxynucleotides by bacteriophage T7 DNA polymerase and *Escherichia coli* DNA polymerase I, (Jun. 1989) Proc Natl. Acad. Sci. USA vol. 86, pp. 4076–4080.
Luis Ugozzoli, et al., Detection of Specific Alleles by Using Allele–Specific Primer Extension Followed by Capture on Solid Support (1991) GATA 9(4): 107–112.
Jon R. Sayers, 5' –3' Exonucleases in phosphorothiate–based oligonucleotide–directed mutagenesis, (1988) vol. 16, No. 3.
David Botstein, et al., Construction of a Genetic Linkage Map in Man Using Restriction Fragment Length Polymorphisms, (1980) Genet 32:314–331/.
Mickey S. Urdea, A novel method for the rapid detection of specific nucleotide sequences in crude biogical samples without blotting or radioactivity; application to the analysis of hepatitis B virus in human serum, (1987) Gene, 61, pp. 253–264.
Kim Holstrøm, A highly Sensitive and Fast Nonradioactive Method for Detection of Polymerase Chain Reaction Products, Analytical Biochemistry 209, 278–283 (1993).
Marjut Ranki, et al., Sandwich hybridization as a convenient method for the detection of nucleic acids in crude, Gene, 21 (1983) 77–85.
George H. Keller, Detection of Hjuman Immunodeficiency Virus Type 1 DNA by Polymerase Chain Reaction Amplification and Capture Hybridization in Microtiter Wells, (Mar. 1991) Journal of Clinical Microbiology, pp. 638–641.
Kenneth L. Beattie, et al., Advances in Genosensor Research (1995) Clin. Chem. 41/5. 700–706.
Jagannath B. Lamture, et al., Direct detection of nucleic acid hybridization on the surface of a charge coupled device (1994) Nucleic Acids Research vol. 22, No. 11 2121–21.
Don I. Stimpson, et al., Real–time detection of DNA hybridization and melting on oligonucleotide arrays using optical wave guides (Jul. 1995) Proc. Natl. Acad. Sci. USA vol. 92, pp. 6379–6383.
Linda A. Chrisey, et al. Covalent attachment of synthetic DNA to self–assembled monolayer films (1996) Nucleic Acids Research, vol. 24, No. 15 3031–3039.

ATTACHMENT OF UNMODIFIED NUCLEIC ACIDS TO SILANIZED SOLID PHASE SURFACES

FIELD OF THE INVENTION

The invention relates to a simple, and preferably cost effective, method for immobilizing nucleic acid molecules onto a solid support. The invention further concerns the use of such immobilized molecules in nucleic acid hybridization assays, sequencing by hybridization assays, and genetic analyses and combinatorial analyses involving nucleic acids or proteins for screening applications.

BACKGROUND OF THE INVENTION

The analysis of the structure, organization and sequence of nucleic acid molecules is of profound importance in the prediction, diagnosis and treatment of human and animal disease, in forensics, in epidemiology and public health, and in the elucidation of the factors that control gene expression and development. Methods for immobilizing nucleic acids are often important in these types of analyses. Three areas of particular importance involve hybridization assays, nucleic acid sequencing, and the analysis of genomic polymorphisms.

I. Nucleic Acid Hybridization

The capacity of a nucleic acid "probe" molecule to hybridize (i.e. base pair) to a complementary nucleic acid "target" molecule forms the cornerstone for a wide array of diagnostic and therapeutic procedures.

Hybridization assays are extensively used in molecular biology and medicine. Methods of performing such hybridization reactions are disclosed by, for example, Sambrook, J. et al. (In: *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989)), Haymes, B. D., et al. (In: *Nucleic Acid Hybridization, A Practical Approach*, IRL Press, Washington, D.C. (1985)) and Keller, G. H. and Manak, M. M. (In: *DNA Probes, Second Edition*, Stockton Press, New York, N.Y. (1993)) which references are incorporated herein by reference.

Many hybridization assays require the immobilization of one component. Nagata et al. described a method for quantifying DNA which involved binding unknown amounts of cloned DNA to microtiter wells in the presence of 0.1M $MgCl_2$ (Nagata et al., *FEBS Letters* 183: 379–382, 1985). A complementary biotinylated probe was then hybridized to the DNA in each well and the bound probe measured calorimetrically. Dahlen, P. et al. have discussed sandwich hybridization in microtiter wells using cloned capture DNA adsorbed to the wells (Dahlen, P. et al., *Mol. Cell. Probes* 1: 159–168, 1987). An assay for the detection of HIV-1 DNA using PCR amplification and capture hybridization in microtiter wells has also been discussed (Keller, G. H. et al., *J. Clin. Microbiol.* 29: 638–641, 1991). The NaCl-mediated binding of oligomers to polystyrene wells has been discussed by Cros et al (French patent no. 2,663,040) and very recently by Nikiforov et al. (*PCR Methods Applic.* 3: 285–291, 1994). The cationic detergent-mediated binding of oligomers to polystyrene wells has very recently been described by Nikiforov et al., *Nucleic Acids Res.* 22: 4167–4175.

II. Analysis Of Single Nucleotide DNA Polymorphisms

Many genetic diseases and traits (i.e. hemophilia, sickle-cell anemia, cystic fibrosis, etc.) reflect the consequences of mutations that have arisen in the genomes of some members of a species through mutation or evolution (Gusella, J. F., *Ann. Rev. Biochem.* 55:831–854 (1986)). In some cases, such polymorphisms are linked to a genetic locus responsible for the disease or trait; in other cases, the polymorphisms are the determinative characteristic of the condition.

Such single nucleotide polymorphisms differ significantly from the variable nucleotide type polymorphisms ("VNTRs"), that arise from spontaneous tandem duplications of di- or tri-nucleotide repeated motifs of nucleotides (Weber, J. L., U.S. Pat. No. 5,075,217; Armour, J. A. L. et al., *FEBS Lett.* 307:113–115 (1992); Jones, L. et al., *Eur. J. Haematol.* 39:144–147 (1987); Horn, G. T. et al., PCT Application WO91/14003; Jeffreys, A. J., U.S. Pat. No. 5,175,082); Jeffreys. A. J. et al., *Amer. J. Hum. Genet.* 39:11–24 (1986); Jeffreys. A. J. et al. *Nature* 316:76–79 (1985); Gray, I. C. et al. *Proc. R. Acad. Soc. Lond.* 243:241–253 (1991); Moore, S. S. et al. *Genomics* 10:654–660 (1991); Jeffreys, A. J. et al. *Anim. Genet.* 18:1–15 (1987); Hillel, J. et al. *Anim. Genet.* 20:145–155 (1989); Hillel, J. et al. *Genet.* 124:783–789 (1990)), and from the restriction fragment length polymorphisms ("RFLPs") that comprise variations which alter the lengths of the fragments that are generated by restriction endonuclease cleavage (Glassberg, J., UK patent application 2135774; Skolnick, M. H. et al., *Cytogen. Cell Genet.* 32:58–67 (1982); Botstein, D. et al. *Ann. J. Hum. Genet.* 32:314–331 (1980); Fischer, S. G. et al. (PCT Application WO90/13668); Uhlen, M., PCT Application WO90/11369)).

Because single nucleotide polymorphisms constitute sites of variation flanked by regions of invariant sequence, their analysis requires no more than the determination of the identity of the single nucleotide present at the site of variation; it is unnecessary to determine a complete gene sequence for each patient. Several methods have been developed to facilitate the analysis of such single nucleotide polymorphisms.

Mundy, C. R. (U.S. Pat. No. 4,656,127), for example, discusses a method for determining the identity of the nucleotide present at a particular polymorphic site that employs a specialized exonuclease-resistant nucleotide derivative. A primer complementary to the allelic sequence immediately 3' to the polymorphic site is permitted to hybridize to a target molecule obtained from a particular animal or human. If the polymorphic site on the target molecule contains a nucleotide that is complementary to the particular exonuclease-resistant nucleotide derivative present, then that derivative will be incorporated onto the end of the hybridized primer. Such incorporation renders the primer resistant to exonuclease, and thereby permits its detection. Since the identity of the exonuclease-resistant derivative of the sample is known, a finding that the primer has become resistant to exonucleases reveals that the nucleotide present in the polymorphic site of the target molecule was complementary to that of the nucleotide derivative used in the reaction. The Mundy method has the advantage that it does not require the determination of large amounts of extraneous sequence data. It has the disadvantages of destroying the amplified target sequences, and unmodified primer and of being extremely sensitive to the rate of polymerase incorporation of the specific exonuclease-resistant nucleotide being used.

Cohen, D. et al. (French Patent 2,650,840; PCT Appln. No. WO91/02087) discuss a solution-based method for determining the identity of the nucleotide of a polymorphic site. As in the Mundy method of U.S. Pat. No. 4,656,127, a primer is employed that is complementary to allelic

sequences immediately 3' to a polymorphic site. The method determines the identity of the nucleotide of that site using labeled dideoxynucleotide derivatives, which, if complementary to the nucleotide of the polymorphic site will become incorporated onto the terminus of the primer.

An alternative method, known as Genetic Bit Analysis™ or GBA™ is described by Goelet, P. et al. (PCT Appln. No. 92/15712). The method of Goelet, P. et al. uses mixtures of labeled terminators and a primer that is complementary to the sequence 3' to a polymorphic site. The labeled terminator that is incorporated is thus determined by, and complementary to, the nucleotide present in the polymorphic site of the target molecule being evaluated. In contrast to the method of Cohen et al. (French Patent 2,650,840; PCT Appln. No. WO91/02087) the method of Goelet, P. et al. is preferably a heterogeneous phase assay, in which the primer or the target molecule is immobilized to a solid phase. It is thus easier to perform, and more accurate than the method discussed by Cohen.

Cheesman, P. (U.S. Pat. No. 5,302,509) describes a method for sequencing a single stranded DNA molecule using fluorescently labeled 3'-blocked nucleotide triphosphates. An apparatus for the separation, concentration and detection of a DNA molecule in a liquid sample has been recently described by Ritterband, et al. (PCT patent Application No. WO95/17676).

An alternative approach, the "Oligonucleotide Ligation Assay" ("OLA") (Landegren, U. et al. *Science* 241:1077–1080 (1988)) has also been described as capable of detecting single nucleotide polymorphisms. The OLA protocol uses two oligonucleotides which are designed to be capable of hybridizing to abutting sequences of a single strand of a target. One of the oligonucleotides is biotinylated, and the other is detectably labeled. If the precise complementary sequence is found in a target molecule, the oligonucleotides will hybridize such that their termini abut, and create a ligation substrate. Ligation then permits the labeled oligonucleotide to be recovered using avidin, or another biotin ligand. Nickerson, D. A. et al. have described a nucleic acid detection assay that combines attributes of PCR and OLA (Nickerson, D. A. et al. *Proc. Natl. Acad. Sci.* (*U.S.A.*) 87:8923–8927 (1990). In this method, PCR is used to achieve the exponential amplification of target DNA, which is then detected using OLA. In addition to requiring multiple, and separate, processing steps, one problem associated with such combinations is that they inherit all of the problems associated with PCR and OLA.

Recently, several primer-guided nucleotide incorporation procedures for assaying polymorphic sites in DNA have been described (Komher, J. S. et al., *Nucl. Acids. Res.* 17:7779–7784 (1989); Sokolov, B. P., *Nucl. Acids Res.* 18:3671 (1990); Syvänen, A. -C., et al., *Genomics* 8:684–692 (1990); Kuppuswamy, M. N. et al., *Proc. Natl. Acad. Sci.* (*U.S.A.*) 88:1143–1147 (1991); Prezant, T. R. et al., *Hum. Mutat.* 1:159–164 (1992); Ugozzoli, L. et al., *GATA* 9:107–112 (1992); Nyrén, P. et al., *Anal. Biochem.* 208:171–175 (1993)). These methods differ from GBA™ in that they all rely on the incorporation of labeled deoxynucleotides to discriminate between bases at a polymorphic site. In such a format, since the signal is proportional to the number of deoxynucleotides incorporated, polymorphisms that occur in runs of the same nucleotide can result in signals that are proportional to the length of the run (Syvänen, A. -C., et al., *Amer. J. Hum. Genet.* 52:46–59 (1993)). Such a range of locus-specific signals could be more complex to interpret, especially for heterozygotes, compared to the simple, ternary (2:0, 1:1, or 0:2) class of signals produced by the GBA™ method. In addition, for some loci, incorporation of an incorrect deoxynucleotide can occur even in the presence of the correct dideoxynucleotide (Komher, J. S. et al., *Nucl. Acids. Res.* 17:7779–7784 (1989)). Such deoxynucleotide misincorporation events may be due to the Km of the DNA polymerase for the mispaired deoxy-substrate being comparable, in some sequence contexts, to the relatively poor Km of even a correctly base paired dideoxy-substrate (Kornberg, A., et al., In: DNA Replication, Second Edition (1992), W. H. Freeman and Company, New York; Tabor, S. et al., *Proc. Natl. Acad. Sci.* (*U.S.A.*) 86:4076–4080 (1989)). This effect would contribute to the background noise in the polymorphic site interrogation.

III. Methods of Immobilizing Nucleic Acids to a Solid Phase

Several of the above-described methods involve procedures in which one or more of the nucleic acid reactants are immobilized to a solid support. Currently, 96-well polystyrene plates are widely used in solid-phase immunoassays, and several PCR product detection methods that use plates as a solid support have been described. The most specific of these methods require the immobilization of a suitable oligonucleotide probe into the microtiter wells followed by the capture of the PCR product by hybridization and colorimetric detection of a suitable hapten. It would be desirable to have an improved immobilization method that could be used to bind oligonucleotides to polystyrene such that their capacity to be used for hybridization, sequencing, or polymorphic analysis would be retained, and which would be rapid, convenient to use and inexpensive. The present invention provides such an improved method.

The means by which macromolecules bind non-covalently to polystyrene and glass surfaces is not well understood. Nevertheless, these adsorption phenomena have proven to be important in the development and manufacturing of immunoassays and other types of diagnostic tests where one component needs to be immobilized.

Polystyrene is a very hydrophobic material because it normally contains no hydrophilic groups. Microtiter plate manufacturers have developed methods of introducing such groups (hydroxyl, carboxylate and others) onto the surface of microwells to increase the hydrophilic nature of the surface. Theoretically, this allows macromolecules to bind through a combination of hydrophobic and hydrophilic interactions (Baier et al., *Science* 162: 1360–1368 (1968); Baier et al., *J. Biomed. Mater. Res.* 18: 335–355 (1984); Good et al., in L. H. Lee (ed.) *Fundamentals of Adhesion*, Plenum, New York, chapter 4 (1989)) (FIG. 1). In practice, some proteins do bind more efficiently to the treated hydrophilic polystyrene than to the untreated material. Covalent binding to polystyrene, especially microtiter wells, has however proven to be difficult, so passive adsorption remains the most commonly used method of binding macromolecules to such wells. The term "polystyrene" may also be used to describe styrene-containing copolymers such as: styrene/divinyl benzene, styrene/butadiene, styrene/vinyl benzyl chloride and others.

While polystyrene is an organic hydrophobic substrate, glass provides an inorganic hydrophilic surface. The most common glass format in immunoassays is the microscope slide. Laboratory-grade glasses are predominantly composed of SiO_2, but they also may contain B_2O_3, Al_2O_3 as well as other oxides (FIG. 2). Interfaces involving such materials have thus become a dynamic area of chemistry in

which surfaces have been modified in order to generate desired heterogeneous environments or to incorporate the bulk properties of different phases into a uniform composite structure. Our purpose here then is to use organosilanes for tailoring surfaces with chemically reactive groups mercapto (SH) and/or epoxy.

While numerous methods for the attachment of oligonucleotides and proteins on surfaces have been described, the methods are both expensive and time consuming. The reported covalent attachments of pre-made oligonucleotides onto modified glass surfaces have been always using modified oligonucleotides in order to increase reactivity and selectivity of oligonucleotides towards surfaces. Typical modifications involved the introduction of amino groups, or thio groups into 3'- and/or 5'-oligonucleotides. For example, Stimpson et al. (*P.N.A.S.* 92:6379–6383 (1995)) reported covalent attachment of 3'-amino oligonucleotides onto epoxy silanized surfaces with acid catalysis but with only 1/10 the density achieved in this invention. Beattie et al. (*Clin. Chem.* 41:700–706 (1995)) reported attachment of 3'- and/or 5'- amino oligonucleotides onto epoxy silanized surfaces under elevated temperature. Lamture et al. (*Nucleic Acids Res.* 22:2121–2125 (1994)) reported the methods for attaching 3'-amino-oligonucleotides onto epoxy silanized slides under 0.1M KOH. Hetero bifunctional cross-linkages have been used to couple the 3' or 5'-thio-modified oligonucleotides or amino-modified onto amino-propyl silanized surfaces as reported by Chrisey et al. (*Nucleic Acids Res.* 24: 303103039 (1996)) and Guo et al. (*Nucleic Acids Res.* 22: 4556–5465 (1994)). All of these reported methods however, require modified oligonucleotides.

The present invention describes a novel method for immobilizing nucleic acid molecules to a solid-phase by means of a covalent ether or thioether linkage. This simple, two-step method has the specificity and efficiency needed to prepare DNA arrays.

SUMMARY OF THE INVENTION

The present invention provides an improved immobilization method that permits the rapid, and inexpensive immobilization of nucleic acid molecules to a solid phase. The invention allows immobilization of oligonucleotides by incubation with a silane-containing or silane-treated solid phase. The immobilized molecules can be used for nucleic acid hybridization assays, sequencing hybridization assays, genetic analyses, combinatorial analyses involving nucleic acids or proteins, and other screening applications such as protein-DNA binding assays.

In detail, the invention provides a method for immobilizing a nucleic acid molecule to a solid phase, the method comprising the steps of:

(A) coating said solid phase with a silane and allowing said silane-coated solid phase to cure; and

(B) coupling unmodified nucleic acid molecules having either a terminal 3' OH or a terminal 5' OH to said silane-coated solid phase.

The invention particularly concerns the embodiments of the above method wherein, in step A, the silane is selected from the group consisting of 3-mercapto-propyl-trimethoxysilane and 3-glycidoxy propyl-trimethoxysilane.

The invention further pertains to oligonucleotide coated surfaces useful in genetic analysis and other screening applications such as protein-DNA binding assays.

The invention particularly concerns oligonucleotide arrays comprising epoxy- or mercapto-silane coated surfaces and unmodified oligonucleotides covalently attached to the epoxy or mercapto-silane, such arrays being useful for nucleic acid hybridization assays, sequencing hybridization assays, genetic analyses, combinatorial analyses involving nucleic acids or proteins, and other screening applications such as protein-DNA binding assays. The features of the coated surface enable standardized patterning of unique oligonucleotides onto silane surface coatings.

The invention further pertains to simultaneous patterning of multiple DNA probes in a high density and in a variety of array formats.

DETAILED DESCRIPTION OF THE INVENTION

I. The Immobilization of Nucleic Acid Molecules

The present invention concerns a method for immobilizing nucleic acid molecules onto a solid-phase. Recently, several methods have been proposed as suitable for immobilizing an oligonucleotide to a solid support. Holmstrom, K. et al., for example, exploit the affinity of biotin for avidin and streptavidin, and immobilize biotinylated nucleic acid molecules to avidin/streptavidin coated supports (Holmstrom, K. et al., *Anal. Biochem.* 209:278–283 (1993)). Another method requires the precoating of the polystyrene or glass solid phases with poly-L-Lys or poly L-Lys, Phe, followed by the covalent attachment of either amino- or sulfhydryl-modified oligonucleotides using bi-functional crosslinking reagents. Unlike the present invention, both methods require the use of modified oligonucleotides as well as a pretreatment of the solid phase; however, the present invention discloses a method to covalently bond oligonucleotides with an "unmodified" 5' or 3'—OH to a solid surface. As used herein, the term "unmodified" refers to the absence of any requirement for specialized reactive groups. It does not refer to the exclusion of nucleotides or oligonucleotides that have such groups, or that are biotinylated, flouresceinated, etc.

Kawai, S. et al. describes an alternative method in which short oligonucleotide probes were ligated together to form multimers and these were ligated into a phagemid vector (Kawai, S. et al., *Anal. Biochem.* 209:63–69 (1993)). The oligonucleotides were immobilized onto polystyrene plates and fixed by UV irradiation at 254 nm. A method for the direct covalent attachment of short, 5'-phosphorylated primers to chemically modified polystyrene plates ("Covalink" plates, Nunc) has also been proposed by Rasmussen, S. R. et al., (*Anal. Biochem.* 198:138–142 (1991)). The covalent bond between the modified oligonucleotide and the solid phase surface is created by a condensation reaction with a water-soluble carbodimide. The Rasmussen method claims a predominantly 5'-attachment of the oligonucleotides via their 5'-phosphates; however, it requires the use of specially prepared, expensive plates. The method of the present invention departs from such methods, in not requiring such unstable and difficult to manipulate crosslinking reagents.

Maskos, U. et al. describes a method to synthesize oligonucleotides directly onto a glass support (Maskos, U. et al., *Nucl. Acids Res.* 20:1679–1684 (1992)). According to this method, a flexible linker with a primary hydroxyl group is bound to the solid support via a glycidoxypropyl silane, wherein the primary hydroxyl group serves as the starting point for the oligonucleotide synthesis.

Numerous methods for the attachment of oligonucleotides and proteins on surfaces have been described. The reported covalent attachments of premade oligonucleotides onto glass surfaces have been always using modified oligonucle-

otides in order to increase reactivity and selectivity of oligonucleotides towards surfaces. Typical modifications involved the introduction of amino groups, or thio groups into 3'- and /or 5'- amino modified oligonucleotides onto epoxy surfaces. Stimpson et al. (*P.N.A.S.* 92:6379–6383 (1995)) discloses the generation of DNA chips for use in automated DNA diagnostic applications. To this effect, the Stimpson article discloses the immobilization of 3' amino-linked oligonucleotides into an organized array on a glycidoxypropyl silane treated glass slide.

Lamture et al. (*Nucleic Acids Res.* 22:2121–2125 (1994)) describes the immobilization of 3' amino modified oligonucleotides to a 3-glycidoxypropyl-trimethoxysilane coated silicon wafer in the presence of 0.1M KOH. The oligonucleotides are covalently immobilized to the solid support by means of a secondary amine linkage. Beattie et al. (*Clin. Chem.* 41:700–706 (1995)) discloses the immobilization of either 3' and/or 5' amino modified oligonucleotides to 3-glycidoxypropyltrimethoxysilane coated glass slides at a temperature of 60° C. Additionally, several other references generally related to the immobilization of oligonucleotides to a solid-support. Chrisey et al. (*Nucleic Acids Res.* 24:3031–3039 (1996), Guo et al. (*Nucleic Acids Res.* 22:45456–5465 (1994), Fahy et al. (*Nucleic Acids Res.* 21:1819–1826 (1993), Sliwkowski et al. (*Biochem. J.* 209:731–739 (1983) all disclose the immobilization of a 5' or 3' modified oligonucleotide to a solid-support.

The method of the present invention provides three distinct advantages over other covalent attachment chemistries for oligonucleotide array preparation. First, while the above-identified prior art references require that the oligonucleotide be either amino or thiol modified, the present invention discloses a method to covalently bond oligonucleotides with an "unmodified" 5' or 3' —OH to a solid surface. The present invention thus differs from conventional methods for the covalent attachment of oligonucleotides to solid phases in that it permits the covalent attachment of "unmodified" oligonucleotides. Accordingly, the present invention provides several significant improvements over the prior art. In particular, the present invention provides a low-cost, stable method for the covalent attachment of "unmodified" oligonucleotides to a silanized solid-phase wherein the covalently bound oligonucleotides have wide uses in genetic and combinatorial analysis involving nucleic acids or proteins. Covalent attachment of oligonucleotides onto solid phase surfaces is therefore achieved without modification of oligonucleotides, thereby dramatically reducing the cost and eliminating the variation in quality of modified oligonucleotides.

Second, the silanized surface of the present method provides a very hydrophobic surface which allows oligonucleotide probe droplets to form at specific and localized positions on the solid surface. Thus, for example, multiple probes can be patterned simultaneously on the surface using, for example, a robotic liquid delivery system or an ink-jet printing technique with no cross contamination between probes, even at a high probe density (10,000 probes/cm^2). Accordingly, the process can be easily automated and scaled-up using an off-the-shelf robot or ink-jet printing instrument. Standard covalent attachment chemistries require the use of photolithographic and laser patterning techniques which require multiple masking and lifting steps for high density DNA array preparation.

Third, unlike traditional techniques, the present method does not require the use of expensive crosslinking agents. These crosslinking agents are difficult to use because of their sensitivity to air and humidity. Therefore, the present method provides a new, efficient and inexpensive method for DNA array preparation, and particularly for large scale DNA array preparation.

In the preferred embodiment, this invention describes a method for immobilizing nucleic acids on silane coated solid phase surfaces which is useful in genetic analysis and other screening applications such as protein-DNA binding assays. The invention pertains to oligonucleotide arrays comprising silane coated solid phase surfaces and unmodified or modified oligonucleotides covalently attached to the silane coated solid phase, such arrays being useful for genetic analyses and combinatorial analyses involving nucleic acids or proteins. The features of the coated surface enable standardized patterning of unique oligunucleotides onto silane surface coatings.

In the most preferred embodiment, the invention provides for methods of covalent attachment of unmodified oligonucleotides onto mercapto-silanized surface or epoxy-silanized surfaces with high density and high stability. The ease of preparation of unmodified oligonucleotides coupled with stable ether (epoxy) or thio-ether (mercapto) linkage attachments renders this method the most cost effective, with little or no variation in terms of the quality of oligonucleotides, stability of attachment linkage and consistency in large scale batch to batch manufactures. Additionally, the hydrophobic property of silane surfaces also allows simultaneous patterning of multiple DNA probes in a high density and in a variety of array formats. Furthermore, a DNA array that is stable to high salt and denaturing conditions such as DMF, urea and elevated temperatures, has wide uses in miniaturized biotechniques such as genetic testing, sequencing by hybridization and combinatory selection of DNA binding molecules.

The covalent attachment of the present invention can be distinguished from other means of attachment, such as van der Waals interaction and ion-ion interactions. Thus, unlike other attachment means, the covalently immobilized oligonucleotide will not be released from the solid-phase during subsequent wash steps. The covalent attachment generally provides more stable binding than noncovalent attachment under elevated temperatures and upon other chemical treatment; thus, giving more flexibility for use in biochemical processes.

II. The Immobilization of Nucleic Acid Molecules Using Epoxy Chemistry

In a preferred embodiment of the present invention, a selective, highly efficient method is provided which employs an epoxy-based attachment chemistry to covalently attach nucleic acid molecules in an end selective manner to a solid-phase. Oligonucleotides have two free hydroxyl groups at the 5'- and 3'- ends which allow oligonucleotides to undergo chemical and/or enzymatic elongation, ligation and circularization. The differences in steric hindrances between these two end hydroxyl groups have enabled 5'-selective esterification, 5'-selective tritylation and 5'-selective oxidation in controlled conditions. Unmodified oligonucleotides for attachment to solid phases under certain controlled conditions thus have the potential for the 5'—OH to react preferentially over the 3'—OH towards epoxy activated surfaces. End-selective attachment is achieved in epoxy chemistry by taking advantage of differences in steric hindrance between the 5' and 3' end of the oligonucleotide. Accordingly, one could block either terminus (via phosphorylation, etc.) and therefore obtain a directionally oriented attachment between the solid phase and the unblocked terminus.

The covalent bond described in the epoxy-based preferred embodiment of the present invention is a covalent ether linkage. End selective attachment ensures that the full sequence of the immobilized oligonucleotide is accessible for any desired biochemical reaction. Since there is no need for any modification of oligonucleotides, this embodiment dramatically reduces the cost and variation in terms of the quality of oligonucleotides. The epoxy-based attachment chemistry allows attachment of all forms of DNA including pcr products or genomic DNA to the silanized surface. Furthermore, in the case of epoxy-based attachment chemistry, the chemical bonds between the silane layer and oligonucleotides are covalent ether linkages, which are stable to heat, high salt, and elevated temperatures.

III. The Immobilization of Nucleic Acid Molecules Using Mercapto Chemistry

The present invention describes in another preferred embodiment a random, highly efficient method which employs a mercapto-based attachment chemistry to covalently attach nucleic acid molecules in a non-specific manner to a solid-phase. The covalent bond described in this second preferred embodiment of the present invention is a covalent thioether linkage. Because this embodiment relies on non-specific binding, the full sequence of the immobilized oligonucleotide may not be accessible for all desired biochemical reactions. Since the mercapto group is very reactive in terms of radical reactions and easily deionized under lower pH (pH 9), this allows a variety of reactions to occur with nucleic acids. Heterocyclic purines (electron rich system stabilizing radicals, particularly at position 7 of purines) and pyrimidines (electron deficient, a nucleophile acceptor) in oligonucleotides are good acceptors for either nucleophilic attack or radical reactions. In mercapto chemistry the highly reactive mercapto groups allow mild conditions for attachment. The resulting array can undergo a variety of biochemical reactions and allows hybridization with high efficiency. The mercapto-based attachment chemistry allows attachment of all forms of DNA including pcr products or genomic DNA to the silanized surface. Furthermore, in the case of mercapto-based attachment chemistry, the chemical bonds between the silane layer and oligonucleotides are covalent thioether linkages, which are stable to heat, high salt, and elevated temperatures.

The unmodified nucleic acid molecules, described in the present invention, may be either genomic DNA (i.e., DNA containing a non-translated region), cDNA (i.e., DNA lacking non-translated regions) or RNA; the nucleic acid molecule may also be either single or double stranded. While any unmodified nucleic acid molecule may be immobilized using the present invention, the preferred nucleic acid molecule of the present invention is an unmodified single-stranded synthetic oligonucleotide. The method for making a synthetic oligonucleotide has been previously described by Gait, M. J. (*Oligonucleotide Synthesis A Practical Approach*, IRL Press Ltd., Oxford (1984)) and Sinha, N. D. et al. (*Nucl. Acids Res.* 12:4539–4557 (1984)) (herein incorporated by reference).

Synthesis of unmodified oligonucleotides of about 10 to about 250 nucleotides in length may be performed on an ABI 392 DNA/RNA synthesizer according to phosphoramidite chemistry.

After synthesis, the unmodified oligonucleotides can be purified (for example, using an HPLC column) to separate the full-length oligonucleotides from any contaminating prematurely terminated (i.e., shortened) oligonucleotides. Prior to use in the coupling reaction, the oligonucleotides are concentrated, and, if desired, the molar concentration of the oligonucleotides can be determined.

Although any of a variety of glass or plastic solid supports can be used in accordance with the methods of the present invention, glass is the preferred support. The support can be fashioned as a bead, dipstick, test tube, pin column, etc. However, an especially preferred support is a glass slide. Alternatively, the solid support can be a form of polystyrene plastic (e.g., 96-well microtiter plate, etc.).

Many different mercaptosilane compounds such as 3-mercapto-propyltrimethoxy-silane, 3-mercaptopropyltriethoxysilane, (mercapto-methyl) dimethoxysilane and (mercaptoethyl)ethyldimethoxysilane, etc. can be used in the present invention for coating the solid support with sulfhydryl groups. The general formula for a mercaptosilane that can be used in this invention is:

$$HS(CH_2)n—SiX_3$$

wherein X is a hydrolyzable group such as alkoxy, acyloxy, amine or halide, etc. All of the mercaptosilanes mentioned above are commercially available from United Chemical, Inc. or Aldrich Chemical Company, Inc.

The silane can be coated onto the solid-phase by any of a number of means. For example, the mercaptosilane can be deposited onto the solid surface as an aerosol or a vapor. Alternatively, the silane can be spread onto the solid-surface by mechanical means (e.g., a spreader bar, a saturated cloth, etc.).

An important feature of the present invention is the hydrophobic nature of silanes. Because of this feature, it is possible for an aqueous solution to form extremely well defined beads on the surface of any solid support coated with mercaptosilane. With an automated delivery system, such as a Hamilton robot or ink-jet printing method, it is possible to form a very complex array of oligonucleotide probes on a mercaptosilane coated glass slide. Such methods can deliver nano to pico-liter size droplets with sub-millimeter spacing. Because the aqueous beads are extremely well defined, it is possible to create an array with an extremely high density of oligonucleotide probes. Thus, it is possible to create arrays having greater than about 10,000 probe droplets/cm^2.

IV. The Use of Immobilized Nucleic Acid Molecules

Immobilized nucleic acid molecules, and more preferably, immobilized oligonucleotides, make an ideal diagnostic tool. Specifically, their versatility and simplicity make them ideal diagnostic tools for the detection of infectious and genetic diseases, mutation analysis, etc.

Although the manner in which the nucleic acid molecules are immobilized to the solid support can be random, one of the preferred embodiments of the invention is to arrange the nucleic acid molecules into an ordered array. As used herein, an array is an orderly arrangement of nucleic acid molecules, as in a matrix of rows and columns. The chemistry of the present invention is such that an individual array can contain either a finite or an infinite number of unique immobilized nucleic acid molecules.

There are two preferred methods to make a nucleic acid array: one is to synthesize the specific oligonucleotide sequences directly onto the solid-phase in the desired pattern (Southern, et al., *Nucl. Acids Res.* 22:1368–1373 (1994); Maskos, et al., *Nucl. Acids Res.* 20:1679–1684 (1992); and Pease, et al., *Proc. Natl. Aced. Sci.* 91:5022–5026 (1994); all of which are herein incorporated by reference) and the other

is to pre-synthesize the oligonucleotides on an automated DNA synthesizer (such as an ABI 392 and then attach the oligonucleotides onto the solid-phase at specific locations (Lamture, et al., *Nucl. Acids Res.* 22:2121–2125 (1994) and (Smith, et al., *Nucl. Acids Res.* 22:5456–5465 (1994) both herein are incorporated by reference). In the first method, the efficiency of the coupling step of each base will affect the quality and integrity of the nucleic acid molecule array. This method generally yields a large percentage of undesired incomplete (shortened) sequences which can create problems in the analysis step and effect the integrity of the analysis. Thus, the quality and integrity of an array synthesized according to the first method is inversely proportional to the length of the nucleic acid molecule. Specifically, the synthesis of longer oligonucleotides results in a higher percentage of incomplete, shortened sequences.

A second, more preferred, method for nucleic acid array synthesis utilizes an automated DNA synthesizer for DNA synthesis. The controlled chemistry of an automated DNA synthesizer allows for the synthesis of longer, higher quality DNA molecules than is possible with the first method. Also, the nucleic acid molecules synthesized according to the second method can be purified prior to the coupling step. Therefore, the quality of the nucleic acid molecule array can be expected to be much higher than the quality of the nucleic acid array of the first method. However, a simple, effective and specific oligonucleotide coupling chemistry is lacking for the attachment of presynthesized oligonucleotides. The present invention describes a simple, effective and efficient method for coupling a pre-synthesized unmodified oligonucleotide onto a solid-phase by means of either an ether or thioether covalent linkage.

A. Hybridization Detection Of PCR Products

Thus, for example, covalently immobilized nucleic acid molecules may be used to detect specific PCR products by hybridization where the capture probe is immobilized on the solid phase (Ranki et al, Gene 21: 77–85 (1983); Keller et al., J. Clin. Microbiol. 29: 638–641 (1991); Urdea et al., Gene 61: 253–264 (1987). A preferred method would be to prepare a single-stranded PCR product before hybridization. A sample, suspected to contain the target molecule, or an amplification product thereof, would then be exposed to the solid-surface and permitted to hybridize to the bound oligonucleotide.

The methods of the present invention do not require that the target nucleic acid contain only one of its natural two strands. Thus, the methods of the present invention may be practiced on either double-stranded DNA, or on single-stranded DNA obtained by, for example, alkali treatment of native DNA. The presence of the unused (non-template) strand does not affect the reaction.

Where desired, however, any of a variety of methods can be used to eliminate one of the two natural stands of the target DNA molecule from the reaction. Single-stranded DNA molecules may be produced using the single-stranded DNA bacteriophage M13 (Messing, J. et al., *Meth. Enzymol.* 101:20 (1983); see also, Sambrook, J., et al. (In: *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989)).

Several alternative methods can be used to generate single-stranded DNA molecules. Gyllensten, U. et al., (*Proc. Natl. Acad. Sci.* (*U.S.A.*) 85:7652–7656 (1988) and Mihovilovic, M. et al., (*BioTechniques* 7:14 (1989)) describe a method, termed "asymmetric PCR," in which the standard "PCR" method is conducted using primers that are present in different molar concentrations. Higuchi, R. G. et al. (*Nucleic Acids Res.* 17:5865 (1985)) exemplifies an additional method for generating single-stranded amplification products. The method entails phosphorylating the 5'-terminus of one strand of a double-stranded amplification product, and then permitting a 5'→3' exonuclease (such as T7 gene exonuclease) to preferentially degrade the phosphorylated strand.

Other methods have also exploited the nuclease resistant properties of phosphorothioate derivatives in order to generate single-stranded DNA molecules (Benkovic et al, U.S. Pat. No. 4,521,509; Jun. 4, 1985); Sayers, J. R. et al. (*Nucl. Acids Res.* 16:791–802 (1988); Eckstein, F. et al., *Biochemistry* 15:1685–1691 (1976); Ott, J. et al., *Biochemistry* 26:8237–8241 (1987)).

Most preferably, such single-stranded molecules will be produced using the methods described by Nikiforov, T. (commonly assigned U.S. Pat. No. 5,518,900, herein incorporated by reference). In brief, these methods employ nuclease resistant nucleotide derivatives, and incorporate such derivatives, by chemical synthesis or enzymatic means, into primer molecules, or their extension products, in place of naturally occurring nucleotides.

Suitable nucleotide derivatives include derivatives in which one or two of the non-bridging oxygen molecules of the phosphate moiety of a nucleotide has been replaced with a sulfur-containing group (especially a phosphorothioate), an alkyl group (especially a methyl or ethyl alkyl group), a nitrogen-containing group (especially an amine), and/or a selenium-containing group, etc. Phosphorothioate deoxyribonucleotide or ribonucleotide derivatives are the most preferred nucleotide derivatives. Methods of producing and using such phosphorothioate derivatives are disclosed by Nikiforov, T. (U.S. Pat. No. 5,518,900).

B. Solid Phase DNA Sequencing

The methods of the present invention may also be used in the practice of solid-phase sequencing as described by Khrapko, K. R. et al. (DNA Seq., 1:375–388 (1991) and Drmanac, R. and Crkvenjakov, R., *Int. T. Genome Res.*, 1: 1–11 (1992)), both herein are incorporated by reference.

C. GBA™ Genetic Bit Analysis

The methods of the present invention may also be used to immobilize the oligonucleotides that are used in the GBA™ Genetic Bit Analysis (Goelet, P. et al., PCT Application No. 92/15712), herein incorporated by reference. GBA™ Genetic Bit Analysis a solid-phase method for the typing of single-nucleotide polymorphisms. Oligonucleotides having a defined sequence complementary to a region that lies immediately proximal or distal to the variable nucleotide of a polymorphism would thus be provided to a polystyrene microtiter well or glass plate, and incubated with a salt, in accordance with the above-described methods.

The immobilized primer is then incubated in the presence of a DNA molecule (preferably a genomic DNA molecule) having a single nucleotide polymorphism whose immediately 3'-distal sequence is complementary to that of the immobilized primer. Preferably, such incubation occurs in the complete absence of any dNTP (i.e. dATP, dCTP, dGTP, or dTTP), but only in the presence of one or more chain terminating nucleotide derivatives (such as a dideoxynucleotide derivative), and under conditions sufficient to permit the incorporation of such a derivative onto the 3'-terminus of the primer. As will be appreciated, where the polymorphic site is such that only two or three alleles exist (such that only two or three species of ddNTPs, respectively, could be incorporated into the primer extension product), the presence of unusable nucleotide triphosphate(s) in the reaction is

immaterial. In consequence of the incubation, and the use of only chain terminating nucleotide derivatives, a single dideoxynucleotide is added to the 3'-terminus of the primer. The identity of that added nucleotide is determined by, and is complementary to, the nucleotide of the polymorphic site of the polymorphism.

Using the method described in the present patent application, oligonucleotide primers can be immobilized on solid phases like polystyrene or glass, hybridized to PCR-derived, single-stranded templates, and subjected to enzymatic extension at their 3'-ends by a single, labeled ddNTP. The nature of the incorporated ddNTP is determined by the nucleotide that is located in the opposite strand (the polymorphic nucleotide). This assay can be conveniently carried out both in polystyrene ELISA plates, or on glass slides.

In this embodiment, the nucleotide of the polymorphic site is thus determined by assaying which of the set of labeled nucleotides has been incorporated onto the 3'-terminus of the bound oligonucleotide by a primer-dependent polymerase. Most preferably, where multiple dideoxynucleotide derivatives are simultaneously employed, different labels will be used to permit the differential determination of the identity of the incorporated dideoxynucleotide derivative.

D. Ligase-Mediated GBA™

The methods and reagents of the present invention can also be used in concert with a polymerase/ligase mediated polymorphic interrogation assay. This assay, termed ligase-mediated GBA™ genetic bit analysis, is a more specific version of the GBA™ genetic bit analysis assay. The additional specificity arises from the addition of a second hybridization step and a ligation step.

In this assay, two oligonucleotides are employed. The first oligonucleotide is a primer that is complementary to the immediately 3'-distal invariant sequence of the polymorphism. The 3'-end of the oligonucleotide is attached to the plate. A second linker oligonucleotide is complementary to the 5'-proximal sequence of the polymorphism being analyzed, but is incapable of hybridizing to the first oligonucleotide. The second linker oligonucleotide is phosphorylated at both its 3' and 5' ends.

These oligonucleotides are incubated in the presence of DNA containing the single nucleotide polymorphism that is to be analyzed, and at least one 2'-deoxynucleotide 5'-triphosphate. The incubation reaction further includes a DNA polymerase and a DNA ligase. The tethered and soluble oligonucleotides are thus capable of hybridizing to the same strand of the target molecule under analysis. The sequence considerations cause the two oligonucleotides to hybridize to the proximal and distal sequences of the single nucleotide polymorphism (SNP) that flank the variable nucleotide of the polymorphism, and to be separated by a single nucleotide at the precise position of the variability.

The presence of a polymerase and the 2'-deoxynucleotide 5'-triphosphate complementary to the nucleotide present in the variable site of the polymorphism permits the extended primer to be ligated to the bound oligonucleotide, thereby immobilizing the primer. The identity of the polymorphic site that was opposite the single nucleotide can then be determined by any of several means. In a preferred embodiment, the 2'-deoxynucleotide 5'-triphosphate of the reaction is labeled, and its detection thus reveals the identity of the complementary nucleotide of the polymorphic site. Several different 2'-deoxynucleotide 5'-triphosphates may be present, each differentially labeled. Alternatively, separate reactions can be conducted, each with a different 2'-deoxynucleotide 5'-triphosphate. In an alternative sub-embodiment, the 2'-deoxynucleotide 5'-triphosphates are unlabeled, and the soluble oligonucleotide is labeled. In this embodiment, the primer that is extended is immobilized on the polystyrene. Separate reactions are conducted, each using a different unlabeled 2'-deoxynucleotide 5'-triphosphate.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features herein before set forth and as follows in the scope of the appended claims.

Having now generally described the invention, the same will be more readily understood through reference to the following examples which are provided by way of illustration, and are not intended to be limiting of the present invention unless specified. All patents, patent applications and publications mentioned herein are incorporated by reference in their entirety.

EXAMPLE 1

EPOXY-BASED CHEMISTRY

Attachment was obtained by a two-step process of silane treatment and oligonucleotide binding. Glass slides were etched in 25% aqueous ammonium hydroxide, rinsed in milliQ water, then in 95% ethanol. They were treated for about 30 minutes in 3-glycidoxy propyl ethanol (95% ethanol, pH 4.5). Slides were cured at 65° C. for at least 12 hours. 2.5–10 uM conc. of oligonucleotides in an alkaline solution (12.5 mm NaOH or KOH) were applied onto cured slides in any desired array formats, in a covered chamber overnight, after which they were stored in water or a covered chamber for later use. A functional test with hybridization and enzymatic reactions gave the desired signal with 400>1 signal to noise ratio by ELF indirect detection reading and radioisotope quantitation (FIG. 1). FIG. 1 shows the results of GBA functional assay of epoxy-based attachment chemistry. The 25-mer primer with (T)10 spacer arm at 5' was used for attachment. Standard GBA conditions were used in terms of nucleoside triphosphate concentration and enzyme concentration and reaction conditions. Synthetic template 1 was designed to give an "A" signal and synthetic template 2 was designed to give a "T" signal. Single base extension (GBA signal) was obtained only with appropriate template, by using ELF indirect detection and also by CCD camera imaging. The signal to noise ratio (S/N) was greater than 400:1.

Additionally, an attachment experiment was designed with the following end-blocked oligonucleotides 5'-end blocked, 3'-end blocked, or both end blocked. The end blocks were achieved by phosphorylation of the end hydroxyl groups during automated DNA synthesis. The attachment for each type of oligonucleotides was quantified by phosphor image analysis and then the ratio of end selectivity was determined. The 5'-end selective attachment of unmodified oligonucleotides to epoxy silanized surfaces was demonstrated with a selectivity ratio of at least 5:1. Attachment through heterocyclic aminos was minimal in certain conditions tested (Table 1).

TABLE 1

Oligo conc. used for att. (uM)	10	5	2.5	1.25
5'-att. (incl. middle)pmole/7mm2	1.0	1.6	1.4	0.9
3'-att. (incl. middle)pmoles/7mm2	0.143	0.29	0.42	0.48
middle att. pmole/7mm2	0.13	0.09	0.35	0.235
5'-att./3'-att.(after sub middle)	87	7.55	15	21
5'-att./middle att.	6.7	16	3	2.8
3'att./middle att.	0.077	2.2	0.2	1.04
3'-& 5-att./middle att.	6.8	19	3.2	3.8

Table 1 shows the end selective attachment of un-modified oligonucleotides by epoxy-based chemistry. Attachment was performed on an epoxy silanized slide with various concentrations of oligonucleotides in 12.5 mM NaOH for overnight and washed sequentially with TNTw and 50 mM NaOH. 5'-att refers to the attachment reading of 3'-phosphorylated oligonucleotides; 3'-att refers to the attachment reading of 5'-phosphorylated oligonucleotides. Middle att refers to the heterocyclic amino attachment reading of both 3'- and 5'-end blocked oligonucleotides. The ^{32}pisotopes images were analyzed on phosphor image quanta software. Attachment at a concentration of 10 uM gave the best selectivity ratio of 87:1 of 5'-end vs. 3'-end attachment. Attachment at concentration of 5 uM gave the best end selectivity ratio of 19:1 of 3'-plus 5'-vs. middle attachment. These results were based on one slide only, however, a number of slides expressed a trend of optimum concentration at 2.5–5 uM for 5'-end selective attachment. In addition, epoxy-silanized surfaces are air-, moisture-, and heat-stable and showed no nonspecific binding to proteins and oligonucleotides under conditions tested. In summary, 5'-end selective attachment of oligonucleotides was demonstrated without modification of oligonucleotides.

EXAMPLE 2

MERCAPTO-BASED CHEMISTRY

Attachment was obtained by a two-step process of silane treatment and oligonucleotide binding. Glass slides were etched in 25% aqueous ammonium hydroxide, rinsed in milliQ water, then in 95% ethanol. They were treated for about 30 minutes in 3-mercapto-propyl-trimethoxysilane (MPTS). Slides were cured for at least 24 hours under dry inert gas (Ar or N_2). 2.5–10 uM conc. of oligonucleotides in an alkaline solution were applied onto cured slides in any desired array formats, in a covered chamber for an overnight, after which they were stored in water or a covered chamber for later use. A functional test with hybridization and enzymatic reactions gave the desired signal (FIG. 2). FIG. 2 shows the results of mercapto-based attachment chemistry and functional assay by hybridization and GBA. The data was based on phosphor imaging quanta analysis. The X axis represents the input concentration of oligonucleotides used for attachment from 0.3125 uM to 40 uM. Oligo attached is represented by the triangle (pmole/7 mm^2), the diamond represents hybridization and the square represents GBA signals (ddATP extension). ^{32}P labeled oligonucleotides were used for determining the attachment density. The ^{32}P 5'-template was used for assessing the hybridization efficiency. The GBA efficiency was determined by incorporation of ^{32}P-ddATP with exo-klenow DNA polymerase without the presence of other cold dideoxynucleotides. The GBA efficiency and hybridization reached saturation at the attachment input of 10 uM.

Additionally, experiments were performed for attachment of dye-labeled nucleotides to mercapto propyl silanized slides which showed stronger signals for A and G and weaker signals for C and T, which favors the hypothesis that radical mechanisms are more involved than nucleophilic attacks. The attachment of ^{32}P labeled oligonucleotides and fluorescein labeled oligonucleotides was demonstrated and quantified (FIG. 2). The attachment results achieved in terms of attachment density, hybridization and genetic bit analysis efficiency were compatible with epoxy chemistry and chemistries reported by others.

EXAMPLE 3

THE RELATIONSHIP BETWEEN PRIMER DENSITY AND HYBRIDIZATION EFFICIENCY

Hybridization efficiency is positively related to the surface density of the attached primer. In this study, increasing amounts of the BRAC1 primer 5' $(T)_{10}$, TCA TTA ATG CTA TGC AGA AAA TCT TAG (SEQ ID No. 1) are covalently attached to a solid surface according to the methods described above. Table 2 shows the results for epoxy-based attachment vs. hybridization efficiency. The data was based on phosphor imaging quanta analysis. 3'-phosphorylated oligonucleotides were used for attachment and 5'-phosphorylated templates were used for hybridiation. A low surface coverage would presumably yield a corresponding low hybridization signal. Conversely, high surface densities might result in steric interference between the covalently immobilized oligonucleotides thereby impeding access to target DNA. The results indicated that with higher coverage up to 1.78 pmoles/7 mm^2, a higher hybridization efficiency was obtained. Increased primer density is associated with increased hybridization efficiency. Accordingly, hybridization efficiency is affected by the stability of the primer attachment.

TABLE 2

conc.for att.	covalent att	Hybridization
40 uM	0.19	0.1 + 0.015
20	0.42	0.14 + 0.00
5	1.78	0.58 + 0.005
2.5	1.61	0.43 + 0.03
1.25	0.98	0.29 + 0.05
0.625	0.42	0.17 + 0.01

EXAMPLE 4

GENETIC BIT ANALYSIS COMPATIBILITY

A GBA primer having a poly-T_{10} residue long spacer arm is attached to the glass surface by means of the previously described epoxy-based chemistry or mercapto-based chemistry. Standard GBA biochemistry is used to analyze two synthetic templates. Each synthetic template is hybridized to GBA primer immobilized to the treated glass slide and treated with an extension mix containing all of the extension reaction components, exonuclease-free Klenow fragment of the *E. coli* polymerase and each of four fluorescein-labeled ddNTP's and co-ddNTP's or ^{32}P-ddATP. The signal was recorded by enzyme-mediated fluorescence using a Cytoflour II fluorescent plate reader (FIG. 1) or phosphor image quanta analysis. FIG. 1 shows the results of GBA functional assay of epoxy-based attachment chemistry. The 27-mer primer with (T)10 spacer arm at 5' was used for attachment (SEQ ID No. 1). Standard GBA conditions were used in terms of nucleoside triphosphate concentration and enzyme concentration and reaction conditions. Synthetic template 1

5' ACA CTC TAA GAT TTT CTG CAT AGC ATT AAT (SEQ ID No. 2) was designed to give an "A" signal and synthetic template 2 5' GGA CAC TAA GAT TTT CGT CAT AGC ATT AAT (SEQ ID No. 3) was designed to give a "T" signal. Single base extension (GBA signal) was obtained only with the appropriate template, by using ELF indirect detection and also by CCD camera imaging. The signal to noise ratio (S/N) was greater than 400:1.

DNA Samples. Genomic DNA was isolated using the SDS/Proteinase K procedure (Maniatis, T. Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor (1989)) from peripheral blood nucleated cells of humans or horses enriched from red blood cells by selective lysis accomplished by diluting blood with a three fold volume excess of ACK lysing buffer (0.15M ammonium chloride, 1 mM potassium bicarbonate, 0.1 mM EDTA). Oligonucleotides were prepared by solid-phase phosphoramidite chemistry using an Applied Biosystems, Inc. Model 391 automated DNA synthesizer (Foster City, Calif.). In the case of primers used in Genetic Bit Analysis (GBA) reactions, detritylation was not performed following the final cycle of synthesis and the full-length oligonucleotide was purified using the Applied Biosystems oligonucleotide purification cartridge (OPC) as recommended by the manufacturer. For most PCR reactions, primers were used directly by drying down the de-protection reaction.

Table 3 depicts the results of an experiment employing a synthetic template 15' ACA CTC TAA GAT TTT CTG CAT AGC ATT ATT (SEQ ID No. 2)(designed to give a GBA signal in base A) and a synthetic template 2, 5' GGA CAC TAA GAT TTT CGT CAT AGC ATT AAT (SEQ ID No. 3) (designed to give a signal in base T). The primer used was $(T)_{10}$ TCA TTA ATG CTA TGC AGA AAA TCT TAG (SEQ ID No. 1). Both signals give strong signals in the expected bases with virtually no noise observed in the other bases (the Signal to Noise Ratio ranged from 520:1 to 600:1).

TABLE 3

	Fluorescent Counts	
Nucleotide Inserted	Template 1	Template 2
A	~600	1
C	ND	ND
G	ND	ND
T	1	~520

EXAMPLE 5

PREPARATION OF AN OLIGONUCLEOTIDE ARRAY BY INK-JET PRINTING

Another method for the automated delivery of the oligonucleotide solution employs an ink-jet printing technique performed by MicroFab (MicroFab Technologies, Inc., Plano, Tex.). In one experiment, four different spot spacings (center to center) and eight different droplet sizes are tested on the mercaptosilane coated surface using an oligonucleotide labeled at the 3'-terminus with fluorescein. The format of the slides, depicted in Table 4, are as follows:

TABLE 4

Slide	Spot	Row (Row Spacing = 6 mm)		
No.	Spacing	Row 1	Row 2	Row 3
Slide 1	1 mm	5 nl	10 nl	25 nl
Slide 2	0.5 mm	1 nl	2 nl	5 nl
Slide 3	250 μm	250 pl	500 pl	1 nl
Slide 4	125 μm	125 pl	250 pl	N/A

The labeled oligonucleotides are detected using a Molecular Dynamic FluorImager 595. The ink-jet printing technique is a suitable method for the manufacture of oligonucleotide arrays with sub-millimeter spacing and nano to pico-liter droplet sizes. As such, the ink-jet printing technique is suitable for large scale manufacture of oligo arrays.

EXAMPLE 6

PREPARATION OF AND OLIGONUCLEOTIDE ARRAY WITH AN AUTOMATIC PIPETING ROBOT

A Hamilton 2200 automated pipeting robot is used to make arrays of oligonucleotide drops, ranging in size from about 100 nl to about 250 nl, with 1 mm spacing between dots. The small volumes of oligonucleotide solution used with the automated pipeting robot allows for rapid drying of the oligonucleotide drops. As in the ink-jet printing method, a Hamilton robot can be programmed to deliver nano to pico-liter size droplets with sub-millimeter spacing.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features herein before set forth and as follows in the scope of the appended claims.

```
SEQUENCE LISTING

(1) GENERAL INFORMATION:

   (iii) NUMBER OF SEQUENCES: 3

(2) INFORMATION FOR SEQ ID NO:1:
```

-continued

```
    (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 37 base pairs
         (B) TYPE: nucleic acid
         (C) STRANDEDNESS: single
         (D) TOPOLOGY: linear

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TTTTTTTTTT TCATTAATGC TATGCAGAAA ATCTTAG                                  37


(2) INFORMATION FOR SEQ ID NO:2:

    (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 30 base pairs
         (B) TYPE: nucleic acid
         (C) STRANDEDNESS: single
         (D) TOPOLOGY: linear

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

ACACTCTAAG ATTTTCTGCA TAGCATTATT                                          30


(2) INFORMATION FOR SEQ ID NO:3:

    (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 30 base pairs
         (B) TYPE: nucleic acid
         (C) STRANDEDNESS: single
         (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: cDNA

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GGACACTAAG ATTTTCGTCA TAGCATTAAT                                          30
```

What is claimed is:

1. A method for immobilizing an unmodified nucleic acid molecule to a glass or plastic solid phase, which comprises the steps of:

(A) coating said solid phase with a mercapto-silane or an epoxy-silane and allowing said silane-coated solid phase to cure; and

(B) coupling said unmodified nucleic acid molecule having either a terminal 3' OH or a terminal 5' OH to said silane-coated solid phase.

2. The method of claim 1, wherein in step A, said coating occurs in the presence of an acidic buffer of aqueous ethanol for 30 minutes.

3. The method of claim 1, wherein in step A, said silane is selected from the group consisting of mercapto-alkyl-trimethoxysilane and glycidoxyalkyl-silane.

4. The method of claim 1, wherein in step A, said silane is 3-mercaptopropyl-trimethoxysilane.

5. The method of claim 4, wherein in step A, said curing occurs for at least 12 hours in the presence of a dry inert gas consisting of Ar or N_2.

6. The method of claim 4, wherein in step B, said covalent linkage is a covalent thioether linkage.

7. The method of claim 1, wherein in step A, said silane is 3-glycidoxy trimethoxypropyl silane.

8. The method of claim 7, wherein in step A, said curing occurs at a temperature of from 60° C. to 70° C. for 10–14 hours.

9. The method of claim 7, wherein in step B, said covalent linkage is a covalent ether linkage.

10. The method of claim 1, wherein in step B, said coupling occurs in alkaline solution.

11. The method of claim 10, wherein in step B, said alkaline solution comprises from 0.0001M to 5M NaOH.

12. The method of claim 10, wherein in step B, said alkaline solution comprises NaOH, KOH, and LiOH.

13. The method of claim 1, wherein in step B, said nucleic acid molecules comprise oligonucleotides.

14. The method of claim 13, wherein said oligonucleotides have a concentration of from 1.0 to 10 μM.

15. The method of claim 1, wherein in step B, said covalent linkage is selected from the group consisting of covalent ether and thioether linkages.

16. The method of claim 1, wherein said solid phase is glass.

17. The method of claim 16, wherein said glass is a microscope slide.

18. The method of claim 1, wherein said solid-phase is plastic.

19. The method of claim 18, wherein said plastic is polystyrene plastic.

20. The method of claim 19, wherein said polystyrene support is a microwell plate.

21. Ache method of claim 19, wherein said polystyrene support is an array designed to fit into a microwell plate.

22. The method of claim 1, wherein said solid-phase is selected from the group consisting of a bead, a plate, a column, a pin and a dipstick.

23. The method of claim 1, wherein the coupling reaction creates an array of immobilized nucleic acid molecules.

24. The method of claim 1, wherein said immobilized nucleic acid is a polynucleotide and wherein said method optionally further comprises the steps of:

(C) capturing from a solution at least one strand of a specific polynucleotide analyte by hybridization to said immobilized polynucleotide; and

(D) detecting the presence of the captured analyte.

25. The method of claim **24**, wherein step C further comprises the steps of:

(C'(1)) amplifying a specific region of a specific genome using a polymerase chain reaction, said region having a sequence complementary to said immobilized polynucleotide; and

(C'(2)) capturing from solution at least one strand of said amplification product by hybridization to said immobilized polynucleotide; and wherein step D further comprises the step of

(D') detecting the presence of the captured amplification product.

26. The method of claim **25**, wherein said method optionally further comprises the steps of:

(E) incubating a sample of nucleic acid of a target organism, containing a single nucleotide polymorphism in the presence of said immobilized polynucleotide primer and at least one dideoxynucleotide derivative, under conditions sufficient to permit a polymerase mediated, template-dependent extension of said primer, said extension causing the incorporation of a single dideoxynucleotide to the 3'-terminus of said primer, said single dideoxynucleotide being complementary to the single nucleotide of the polymorphic site of said polymorphism;

(F) permitting said template-dependent extension of said primer molecule, and said incorporation of said single dideoxynucleotide; and

(G) determining the identity of the nucleotide incorporated into said polymorphic site, said identified nucleotide being complementary to said nucleotide of said polymorphic site.

27. The method of claim **1**, wherein said coating step is by means of an aerosol, a vaporization means or any other mechanical means.

28. A method for immobilizing an unmodified nucleic acid molecule to a mercapto-silane or epoxy-silane-coated solid phase comprising coupling said unmodified nucleic acid molecule, wherein said nucleic acid molecule has either a terminal 3' OH or a terminal 5' OH, to said silane-coated solid phase.

29. The method of claim **28**, wherein said silane is selected from the group consisting of mercapto-alkyltrimethoxysilane, glycidoxy-alkyl-silane, 3-mercaptopropyl-trimethoxysilane, and 3-glycidoxy propyl trimethoxysilane.

* * * * *

Brief Summary Text - BSTX (53):

The unmodified nucleic acid molecules, described in the present invention, may be either genomic DNA (i.e., DNA containing a non-translated region), cDNA (i.e., DNA lacking non-translated regions) or RNA; the nucleic acid molecule may also be either single or double stranded. While any unmodified nucleic acid molecule may be immobilized using the present invention, the preferred nucleic acid molecule of the present invention is an unmodified single-stranded synthetic oligonucleotide. The method for making a synthetic oligonucleotide has been previously described by Gait, M. J. (Oligonucleotide Synthesis A Practical Approach, IRL Press Ltd., Oxford (1984)) and Sinha, N. D. et al. (Nucl. Acids Res. 12:4539-4557 (1984)) (herein incorporated by reference).

Brief Summary Text - BSTX (56):

Although any of a variety of glass or plastic solid supports can be used in accordance with the methods of the present invention, glass is the preferred support. The support can be fashioned as a bead, dipstick, test tube, pin column, etc. However, an especially preferred support is a glass slide. Alternatively, the solid support can be a form of polystyrene plastic (e.g., 96-well microtiter plate, etc.)

Brief Summary Text - BSTX (42):

The method of the present invention provides three distinct advantages over other covalent attachment chemistries for oligonucleotide array preparation. First, while the above-identified prior art references require that the oligonucleotide be either amino or thiol modified, the present invention discloses a method to covalently bond oligonucleotides with an "unmodified" 5' or 3' --OH to a solid surface. The present invention thus differs from conventional methods for the covalent attachment of oligonucleotides to solid phases in that it permits the covalent attachment of "unmodified" oligonucleotides. Accordingly, the present invention provides several significant improvements over the prior art. In particular, the present invention provides a low-cost, stable method for the covalent attachment of "unmodified" oligonucleotides to a silanized solid-phase wherein the covalently bound oligonucleotides have wide uses in genetic and combinatorial analysis involving nucleic acids or proteins. Covalent attachment of oligonucleotides onto solid phase surfaces is therefore achieved without modification of oligonucleotides, thereby dramatically reducing the cost and eliminating the variation in qual

US005705344A

United States Patent [19]

Giordano et al.

[11] **Patent Number:** **5,705,344**

[45] **Date of Patent:** **Jan. 6, 1998**

[54] **HIGH-THROUGHPUT SCREENING ASSAY FOR INHIBITORS OF NUCLEIC ACID HELICASES**

[75] Inventors: **Heidi Giordano**, Oakland; **Michael G. Peterson**, Millbrae; **Mohanram Sivaraja**, Mountain View, all of Calif.

[73] Assignee: **Tularik, Inc.**, South San Francisco, Calif.

[21] Appl. No.: **616,046**

[22] Filed: **Mar. 14, 1996**

[51] **Int. Cl.**[6] **C12Q 1/68**

[52] **U.S. Cl.** **435/6**; 935/77; 935/78; 435/196

[58] **Field of Search** 135/6, 196; 436/501; 935/77, 78

[56] **References Cited**

U.S. PATENT DOCUMENTS

4,203,848	5/1980	Grandine, II	210/490
5,217,864	6/1993	Heintz et al.	435/6
5,273,881	12/1993	Sena et al.	425/6
5,466,576	11/1995	Schulz et al.	435/6

OTHER PUBLICATIONS

Matson (1986) J. Biol. Chem. 261:10169–75.
Matson and Bean (1995) Methods Enzymol. 262:389–405.
Poll et al. (1994) Biochemistry 33:3841–47.

Crute et al. (1995) J. Med. Chem. 38:1820–25.

Sun and Hurley (1992) J. Med. Chem. 35:1773–82.

Amaratunga et al. (1993) Biochemistry 32:6815–20.

Matson and Kaiser–Rogers (1990) Annu. Rev. Biochem. 59:289–329.

Bachur et al. (1992) Mol. Pharm. 41:993–8.

Schaeffer et al. (1993) Science 260:58–63.

Tuteja et al. (1990) Nucl. Acids Res. 18:6785–92.

Tuteja et al. (1992) Nucl. Acids Res. 20:5329–37.

Maine et al. (1992) Biochemistry 31:3968–75.

Naegeli et al. (1993) Biochemistry 32:613–21.

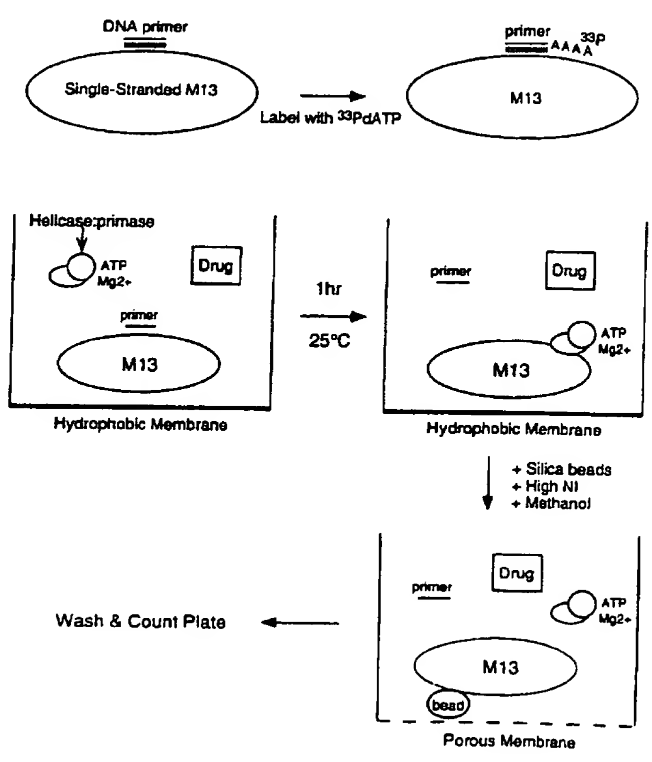
Seki et al. (1990) Biochemistry 29:1003–9.

Primary Examiner—W. Gary Jones
Assistant Examiner—Amy Atzel
Attorney, Agent, or Firm—Richard Aron Osman

[57] **ABSTRACT**

The invention provides screening assays for identifying an inhibitor of a pathogenic helicase activity, e.g. a helicase derived from a pathogenic infectious organism such as a bacterium, protozoan or fungus. Helicase activity is detected by the solid-phase, preferential capture of retained (non-liberated) single-stranded nucleic acid comprising a detectable label.

17 Claims, 4 Drawing Sheets



10 2b

1, 3, 5, 7,
8, 9 (claim 5)

12, 13,
15, 17,
19, 20

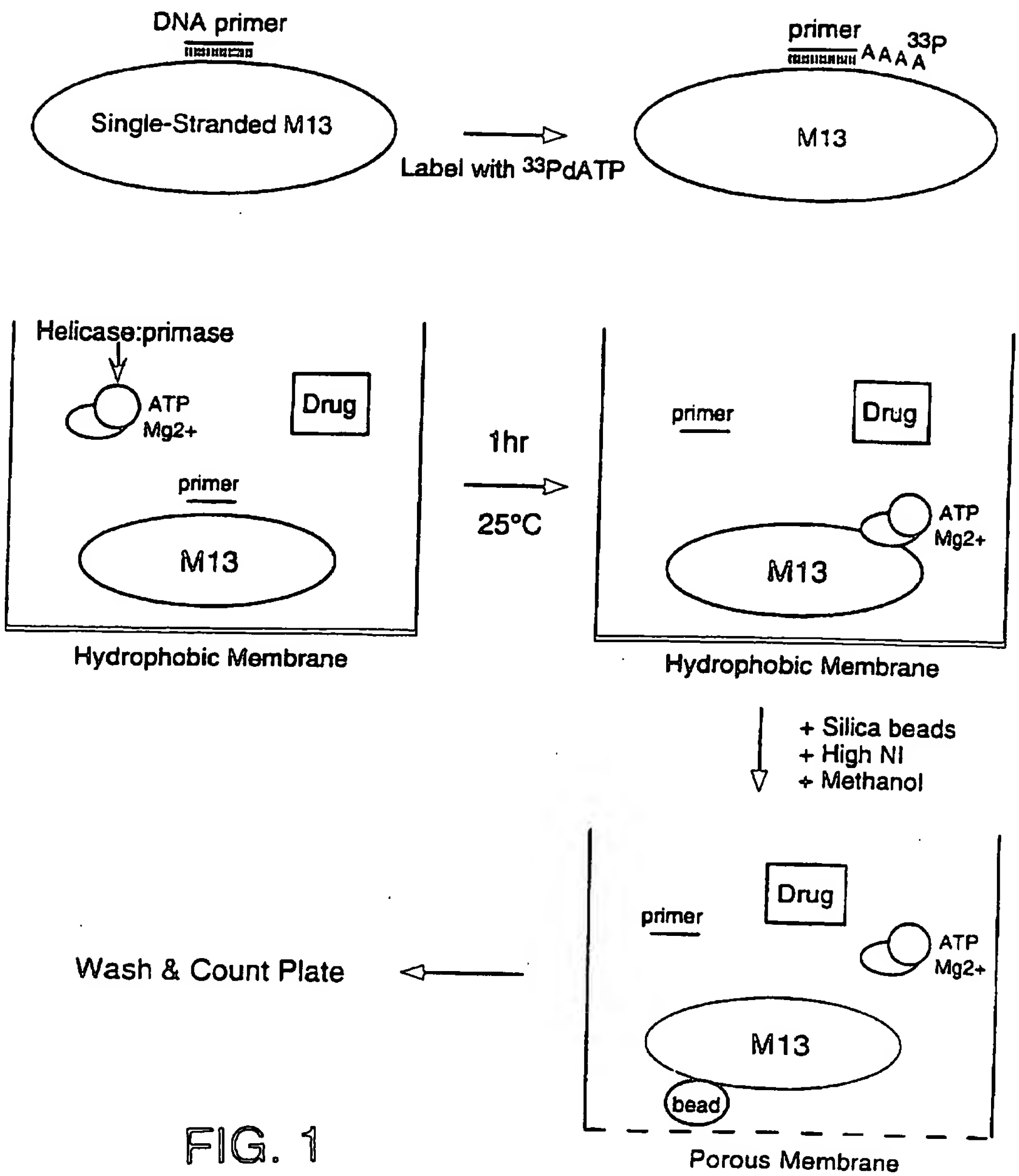

DNA primer
Single-Stranded M13
Label with 33PdATP
primer
AAAA
33P
M13
Helicase:primase
ATP
Mg2+
Drug
primer
M13
Hydrophobic Membrane
1hr
25°C
primer
Drug
ATP
Mg2+
M13
Hydrophobic Membrane
+ Silica beads
+ High NI
+ Methanol
primer
Drug
ATP
Mg2+
M13
bead
Porous Membrane
Wash & Count Plate

FIG. 1

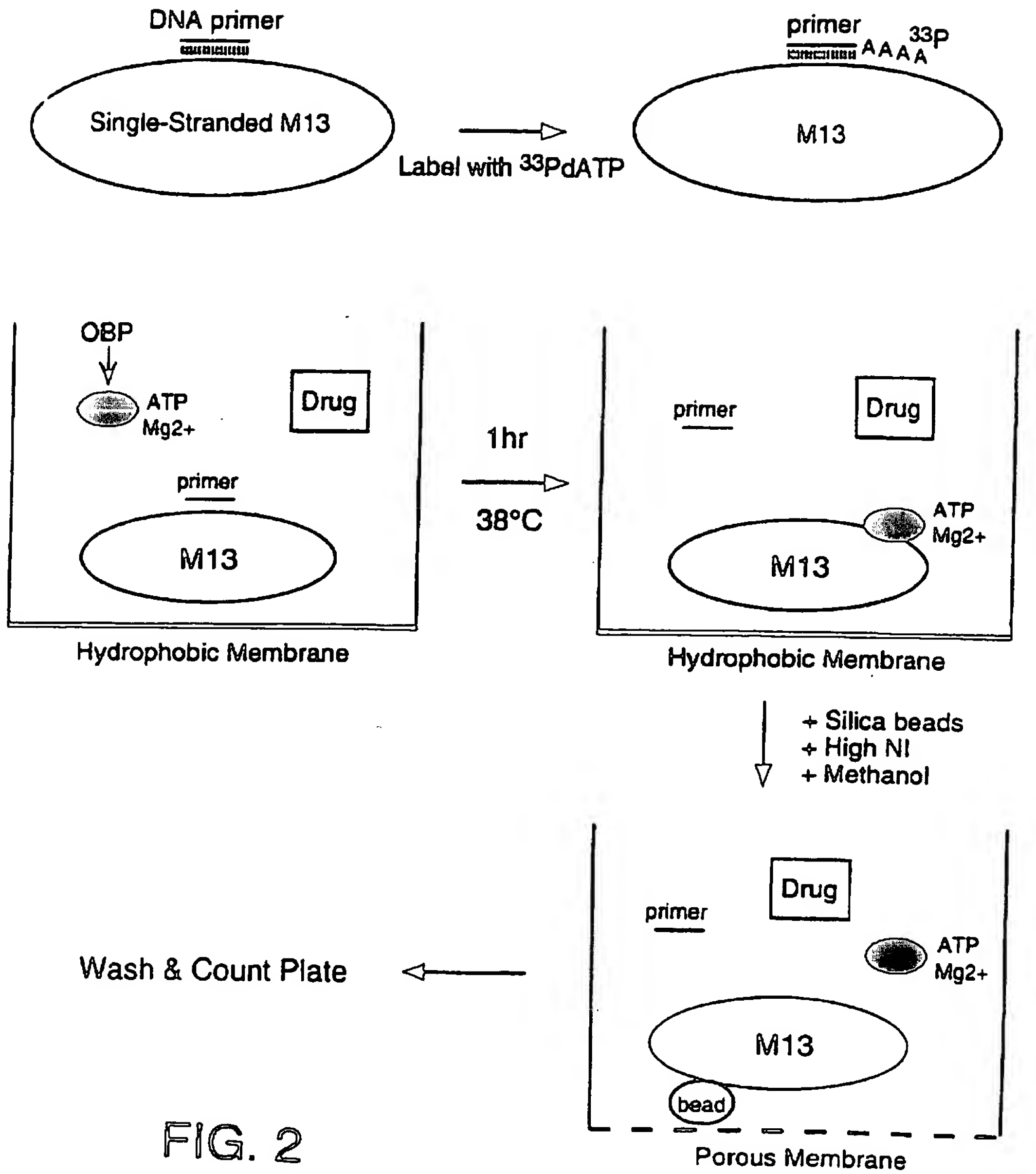

DNA primer
Single-Stranded M13
Label with 33PdATP
primer
AAAA
33P
M13
OBP
ATP
Mg2+
Drug
primer
M13
Hydrophobic Membrane
1hr
38°C
primer
Drug
ATP
Mg2+
M13
Hydrophobic Membrane
+ Silica beads
+ High NI
+ Methanol
Wash & Count Plate
primer
Drug
ATP
Mg2+
M13
bead
Porous Membrane

FIG. 2

FIG.3A
UL5/52 Dose Response

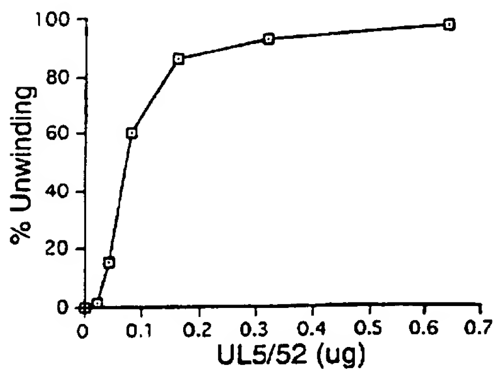


FIG.3B
M13 Template Dose Response

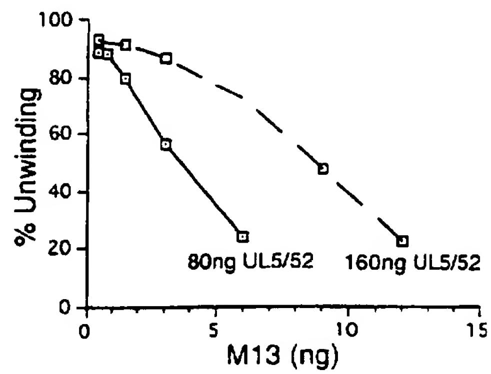


FIG.3C
Effect of Temperature on UL5/52 Activity

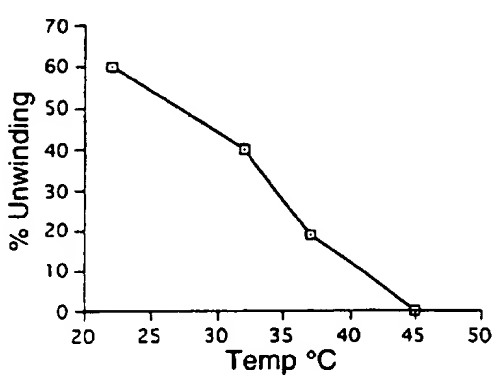


FIG.3D
Effect of pH on UL5/52 Activity

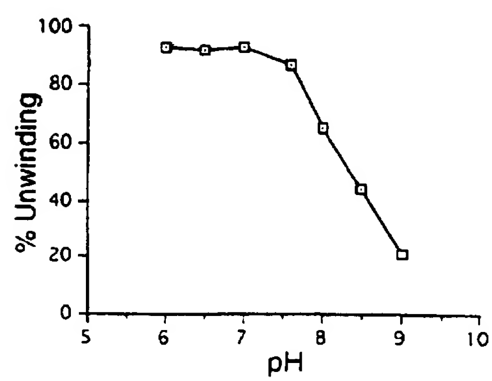


FIG.4A
OBP Dose Response

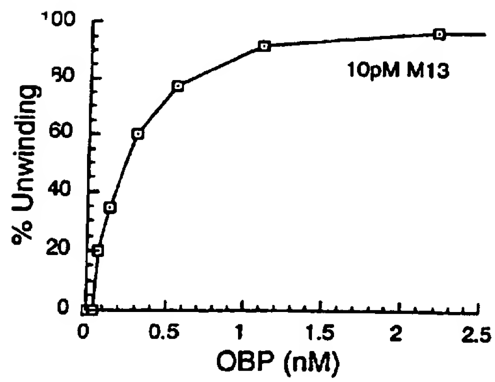


FIG.4B
M13 Dose Response

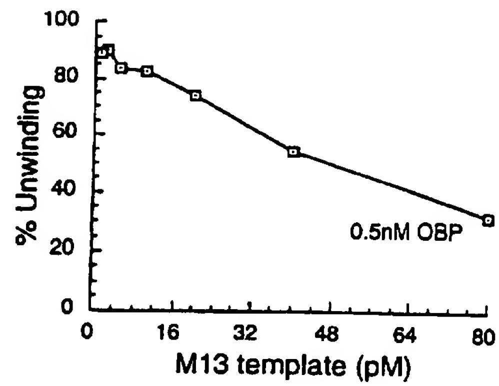


FIG.4C
Effect of Temperature on OBP Activity

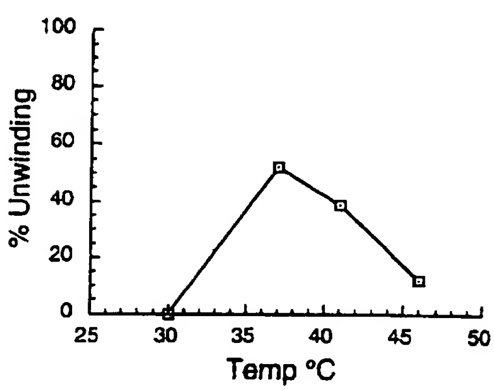
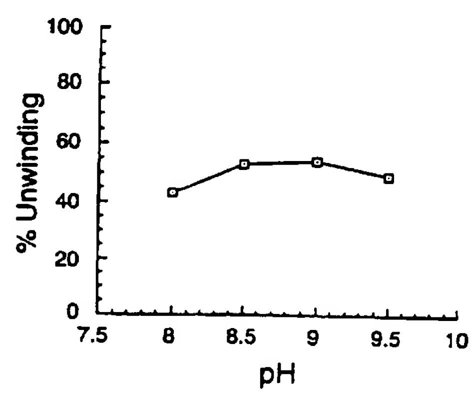


FIG.4D
Effect of pH on OBP Activity



HIGH-THROUGHPUT SCREENING ASSAY FOR INHIBITORS OF NUCLEIC ACID HELICASES

INTRODUCTION

1. Field of the Invention

The field of this invention is assays for screening for inhibitors of a certain class of enzymes, namely nucleic acid helicases.

2. Background

Helicases are enzymes which unwind double-stranded nucleic acids, usually in an NTP-dependent manner. Cellular, microbial, phage, and viral helicases are involved in a wide variety of cellular functions including DNA replication, recombination, and repair and RNA transcription, translation, and processing. Because of the critical functions played by helicases, they provide promising targets for therapeutic intervention, e.g. in pathogenic infection. For example, many infectious diseases, especially fungal and viral disease, have resisted efforts to identify effective pharmaceutical therapies.

Identifying and developing new pharmaceuticals is a multibillion dollar industry in the U.S. alone. Especially needed are efficient methods of identifying pharmacological agents or drugs which are active against pathogens which have hitherto defied effective therapy. If amenable to automated, cost-effective, high throughput drug screening, assays for specific helicase inhibitors would have immediate application in a broad range of domestic and international pharmaceutical and biotechnology drug development programs.

The standard assay for measuring helicase activity uses gel electrophoresis to monitor unwinding of double-stranded DNA. Because this assay is time-consuming and cumbersome, several additional types of helicase assays have been developed: measuring the sensitization of labeled duplex DNA to single-strand specific nucleases, electron microscopy, displacement of a labeled fragment which is annealed to a single-stranded DNA or RNA molecule, and more recently, spectrophotometric assays utilizing a dye or ssDNA binding protein as the reporter molecule. Unfortunately, each of these assays has limitations which restrict their applicability to high-throughput drug screening: they are slow, expensive, insensitive, subject to interference and/or require considerable manipulation.

Relevant Literature

Matson et at. (1990) Annu Rev Biochem 59, 289–329 provides an overview of helicases and helicase assays.

Houston and Kodadek (1994) Proc Natl Acad Sci USA 91, 5471–6474 and Raney et al. (1994) Proc Nail Acad Sci USA 91, 6644–6648 describe spectophotometic helicase assays. Roman and Kowalczykowski (1989) Biochemistry 28, 2863–2873 describes a helicase assay exploiting the intrinsic fluorescence of *E. coli* SSB protein that is quenched when the protein binds single-stranded DNA.

SUMMARY OF THE INVENTION

The invention provides methods and compositions for detecting helicase activity and identifying modulators of pathogenic nucleic acid helicase activity. Helicase activity is detected by the solid-phase, preferential capture of retained (non-liberated) single-stranded nucleic acid comprising a detectable label. The invention includes methods of screening for helicase activity by comparing candidate helicase activities with one or more defined control helicase activities or screening for modulators of one or more defined helicase activities by comparing helicase activities in the presence and absence of a candidate modulator.

The general method for identifying modulators of helicase activity involves steps:

forming a mixture of a labeled first single-stranded nucleic acid comprising a label, an unlabeled second single-stranded nucleic acid, a pathogenic nucleic acid helicase, a nucleoside triphosphate, and a candidate agent, wherein the first and second nucleic acids are hybridized;

incubating the mixture in a reservoir under condition whereby, but for the presence of the candidate agent, the helicase unhybridizes the first and second nucleic acids at a first helicase activity;

immobilizing the second nucleic acid on a solid substrate;

separating any unhybridized first nucleic acid from the second nucleic acid to obtain isolated second nucleic acid;

measuring the mount of the label retained on the isolated second nucleic acid to obtain a second helicase activity;

wherein a difference between the first and second helicase activity indicates that the candidate agent modulates the activity of the pathogenic nucleic acid helicase.

In specific embodiments of the invention, the immobilizing step comprises directly or indirectly (e.g. through an avidin-biotin complex), noncovalently and preferentially binding the second nucleic acid to the solid substrate. For example, the solid substrate may be a glass bead and the preferential affinity resulting from the second nucleic acid being of greater length than the first nucleic acid.

The separating step may involve filtering the solid substrate through a specialized apparatus comprising a tube having a fluid passage comprising a reservoir portion and a evacuation portion, a filter extending transversely across the passage and separating the reservoir and evacuation portions, said filter being changeable from substantially water-impermeable to water-permeable, having a maximum pore size sufficiently large to freely pass said any unhybridized first nucleic acid and sufficiently small to retain said solid substrate; for example, by contacting the filter with an effective concentration of a "wetting agent" such as an organic solvent.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 shows a schematic of an automated Herpes Simplex Virus UL5/52 helicase assay.

FIG. 2 shows a schematic of an automated Herpes Simplex Virus OBP helicase assay.

FIGS. 3A–3D provide a characterization of the Herpes Simplex Virus UL5/52 helicase assay.

FIGS. 4A–4D provide a characterization of the Herpes Simplex Virus OBP helicase assay.

DETAILED DESCRIPTION OF THE INVENTION

The invention provides efficient methods and compositions for detecting helicase activity and identifying specific modulators of nucleic acid helicase activity. The methods are amenable to automated, cost-effective high throughput drug screening and have immediate application in a broad range of domestic and international pharmaceutical and biotechnology drag development programs. In addition, the

invention provides kits for helicase modulator screening which include premeasured amounts of the compositions used in the disclosed methods.

Since helicases are necessary for a wide variety of cellular functions including growth, target diseases are limited only in that disease or disease progression be subject to inhibition by modulation of the activity of one or more specific helicases. As such, target diseases include viral, bacterial and fungal infections, metabolic disease, genetic disease, cell growth and regulatory disfunction, such as neoplasia, inflammation, hypersensitivity, etc. The target diseases may be afflictions of plants, especially agricultural crops, or animals, especially livestock, house animals and humans.

The initial step of the general method involves forming a mixture of a labeled first single-stranded nucleic acid comprising a label, an unlabeled second single-stranded nucleic acid, a pathogenic nucleic acid helicase, a nucleoside triphosphate, and a candidate agent wherein the first and second nucleic acids are hybridized.

The hybridized first and second nucleic acids may be RNA or DNA, linear or circular, depending on the specificity of the targeted helicase. In addition, other nucleic acids or structural analogs may be substituted so long as they provide an active substrate for the targeted helicase activity. The nucleic acids may be of any length amenable to the assay conditions and requirements. For example, ensuring helicase substrate specificity and minimizing non-specific renaturation requires a minimal region of complementarity between the first and second nucleic acids, typically at least about 12, more typically at least about 18 and preferably at least about 24 continuous base pairs. In one embodiment, the differential affinity of the nucleic acids for the solid substrate is a result of a size differential between the strands. In this case, the sizes are selected to maintain differential affinities sufficient to provide statistically significant assay results; frequently the sizes will differ by at least one or two orders of magnitude. In general, optimal lengths are readily determined empirically.

The nucleic acids may be of any sequence which provides a convenient substrate for the targeted helicase(s). The nucleic acids may be complementary over the entire length of at least one of the nucleic acids or there may be regions of noncomplementary 5' and/or 3' of the complementary region. Introducing these 5' and/or 3' noncomplementary regions provides molecular forks that yield better substrates for some helicases. Generally, conveniently replicated vectors e.g. phage, or restriction fragments thereof, provide an inexpensive source of the nucleic acids. The assays are generally compatible with the presence of DNA binding proteins, such as histories. It is often advantageous to include a variety of potential substrates, e.g. double-stranded nucleic acids of varied size, sequence, protein complexing, etc. to improve the likelihood of detecting substrate-sensitive helicases.

The first nucleic acid comprises a detectable label, which label is absent from the second nucleic acid. A wide variety of directly and/or indirectly detectable labels may be used so long as they are compatible with the assay. Exemplary directly detectable labels include radiolabels, fluorescent labels, etc.; exemplary indirectly detectable labels include epitope tags, biotin, nucleoside analogs such as digoxigenin, etc.

labels

The pathogenic helicase (i.e. any helicase activity that is harmful or acting harmfully to the host cell or organism) is selected based on the target application. Rapidly growing cells (e.g. in neoplasia) may be targeted by inhibitors of human helicases, especially replicative helicases. In addition, pathogen-selective or -specific helicases are used to identify pharmacological therapeutics for the treatment of infectious disease. Fungal, viral, bacterial and parasitic helicases, in particular, provide medically urgent targets for identifying inhibitors by the subject methods. Alternatively, a plurality of helicases or panel comprising a preselected range of different helicases can be used to maximize the scope of the assay.

Preferred pathogenic helicases derive from medically significant infectious fungi such as *Aspergillus, Candida species;* bacteria such as *Staphylococci* (e.g *aureus*), *Streptococci* (e.g. *pneumoniae*), *Clostridia* (e.g. *perfringens*), *Neisseria* (e.g *gonorrhoeae*), *Enterobacteriaceae* (e.g. *coli*), *Helicobacter* (e.g *pylori*), *Vibrio* (e.g. *cholerae*), *Capylobacter* (e.g. *jejuni*), *Pseudomonas* (e.g *aeruginosa*), *Haemophilus* (e.g. *influenzae*), *Bordetella* (e.g. *pertussis*), *Mycoplasma* (e.g. *pneumoniae*), *Ureaplasma* (e.g. *urealyticum*), *Legionella* (e.g. *pneumophila*), *Spirochetes* (e.g. *Treponema, Leptospira and Borrelia*), *Mycobacteria* (e.g. *tuberculosis, smegmatis*), *Actinomycies* (e.g. (*israelii*), *Nocardia* (e.g. *asteroides*), *Chlamydia* (e.g. *trachomatis*), Rickettsia, Coxiella, Ehrilichia, Rochalimaea, Brucella, Yersinia, Fracisella, and Pasteurella; *protozoa* such as *sporozoa* (e.g. Plasmodia), *rhizopods* (e.g. Entamoeba) and *flagellates* (Trypanosoma, Leishmania, Trichomonas Giardia, etc.); and viruses such as (+) RNA viruses (examples include Picornaviruses, e.g. polio; Togaviruses, e.g. rubella; Flavivimses, e.g. HCV; and Coronaviruses), (−) RNA viruses (examples include Rhabdoviruses, e.g. VSV; Paramyxovimses, e.g. RSV; Orthomyxovimses, e.g. influenza; Bunyaviruses and Arenaviruses), dsDNA viruses (Reoviruses, for example), RNA to DNA viruses, i.e. Retroviruses, e.g. HIV, and certain DNA to RNA viruses such as Hepatitis B virus.

The helicase may be purified from a natural soume or may be recombinant and is usually provided in at least a partially-purified form. Often only a portion of the native helicase is used in the assay, the portion being sufficient for helicase activity, preferably not less than an order of magnitude less than that of the full-length helicase. Portions capable of imparting the requisite binding specificity and affinity are readily identified by those skilled in the art. A wide variety of molecular and biochemical methods are available for generating catalytic portions, see e.g. Molecular Cloning, A Laboratory Manual (2nd Ed., Sambrook, Fritsch and Maniatis, Cold Spring Harbor), Current Protocols in Molecular Biology (Eds. Aufubel, Brent, Kingston, More, Feidman, Smith and Stuhl, Greene Publ. Assoc., Wiley-Interscience, NY, N.Y., 1992) or that are otherwise known in the art.

The reaction mixture also comprises a candidate agent such as a preselected candidate helicase inhibitor or, especially for high-throughput drug screening, a library-derived candidate agent. Library-derived candidate agents encompass numerous chemical classes, though typically they are organic compounds; preferably small organic compounds. The libraries may comprise synthetic and/or naturally derived compounds. For example, numerous means are available for random and directed synthesis of a wide variety of organic compounds and biomolecules, including expression of randomized oligonucleotides. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural and synthetically produced libraries and compounds are readily modified through conventional chemical, physical, and biochemical means. In addition,

known pharmacological agents may be subject to directed or random chemical modifications, such as acylation, alkylation, esterification, amidification, etc., to produce structural analogs. The agent is provided in standard serial dilutions or in an amount determined by analogy to known modulators.

In addition, the mixture usually includes additional reagents, such as salts, buffers, etc. to facilitate or maximize helicase activity. Also, reagents that reduce non-specific or background denaturation or otherwise improve the efficiency of the assay, such as protease inhibitors, nuclease inhibitors, antimicrobial agents, single-stranded DNA binding protein, etc. may be used.

The second step of the general method involves incubating the mixture in a reservoir under conditions whereby, but for the presence of the candidate agent, the helicase (or helicases) unhybridizes the first and second nucleic acids at a first, control helicase activity and so converts at least a detectable portion, and preferably, substantially all of the initial amount of double-stranded nucleic acid into unhybridized complementary single-stranded nucleic acid, whereby a final amount of the double-stranded nucleic acid remains (i.e. results or is formed). As such, depending on the stop point of the reaction, the measured activity may reflect a catalytic rate or an equilibrium constant. A wide variety of reaction conditions can be employed depending on the targeted helicase(s); in vitro conditions to support activity of exemplary helicases are exemplified below and/or otherwise known in the art. For example, the reaction generally requires the presence of an effective amount of a nucleoside triphosphate, such as ATP. For many helicases pathogenic in mammals, the reaction is carried out at room or elevated temperatures, usually in the range of 20° to 40° C., conveniently at room (ca. 25° C.) temperature. For high-throughput applications, reactions time is minimized, and is usually from 0.1 to 4 hours, more usually about 0.5 to 1.5 hours.

Following incubation, the second nucleic acid is preferentially immobilized on a solid substrate, i.e. the second nucleic acid has a higher affinity for the substrate than does the first. Preferred solid substrates for this capture step maximize the disparity in binding affinities and second nucleic acid binding affinity and binding sites, and hence, maximize signal strength and the signal-to-noise ratio. The affinity for the second nucleic acid may be direct (substrate-nucleic acid), indirect via a ligand (substrate-ligand-nucleic acid) or ligand receptor complex (substrate-receptor-ligand-nucleic acid), etc. As examples: silica-based bead substrates may be used to size-select the nucleic acids directly; magnetized substrates may be used to distinguish second nucleic acid comprising an iron-based ligand; substrates comprising a surface bound antibody receptor may be used to distinguish second nucleic acid comprising a specific ligand antigen of the antibody receptor; etc. To avoid interference, any selected ligand should not be identical to any selected label or label component.

In another embodiment, the hybridized nucleic acids are preferentially immobilized on the solid substrate, i.e. the hybridized nucleic acids have a higher affinity for the substrate than does at least the first nucleic acid, and frequently, both first and second nucleic acids. In this embodiment, the substrate frequently distinguishes double-stranded from single stranded nucleic acid based on differential net charge or charge distribution. An exemplary solid substrate useful in this embodiment is crystalline hydroxyapatite.

Preferred substrate structures includes fine fibers, beads, etc., and in particular, polymeric or silica-based microbeads of size, range and structure to maximize surface area, filter retention and bead suspension time during the assay incubations. Generally, such bead diameters range from 1 to 400 um, usually from 2 to 200 um. Depending on the selected capture mechanism, the mixture may be supplemented with a suitable pH buffer and salt to ensure the ionic strength and pH of the mixture is conducive to optimal binding. For high-throughput applications, the capture incubation is generally less than 4 hours, preferably less than 2 hours, more preferably less than about 1 hour. Typically, capture is most conveniently done at room temperature.

Following immobilization, the solid substrate is washed free of unhybridized first nucleic acid. The method used for separating and washing depends on the nature of the reaction reservoir and the solid substrate. For example, where the substrate is in the form of aggregated fibers, the solid phase may be physically transferred from the reaction reservoir to a series of rinse reservoirs. In a preferred embodiment, the separating and washing steps are performed by filtration, frequently vacuum-assisted filtration. In a particular embodiment, the method uses a filter that is changeable from water-impermeable to water-permeable having a maximum pore size sufficiently small to retain the selected solid substrate, e.g. a maximum pore size less than about 200 um diameter is typically used retain microbeads, by "wetting" the filter, e.g. contacting the filter with an effective concentration of an organic solvent, optionally supplemented with an effective amount of a detergent such as NP-40. In this specific embodiment, typical organic solvents include alcohol, conveniently methanol, solutions in the 5 to 90% range, conveniently about 10% (v/v). The filter should minimize final retention of unhybridized and immobilized labeled first nucleic acid under the assay conditions. A particular embodiment uses a GF/C hydrophobic glass fiber membrane (Polyfiltronics, Rockland, Mass.), or alternatively, a GF/C hydrophilic glass fiber membrane (Polyfiltronics, Rockland, Mass.) superimposed with an MP PP hydrophobic polypropylene membrane (Polyfiltronics, Rockland, Mass).

After washing, the amount of label retained on the substrate is measured to infer the helicase activity in the presence of the candidate agent, wherein a difference between the activity in the presence and absence of the agent indicates that said candidate agent modulates the activity of the targeted helicase. A variety of methods may be used to detect the substrate-bound label depending on the nature of the label and other assay components. Labels may be directly detected through optical or electron density, radiative emissions, nonradiative energy transfers, etc. In the preferred case of radioactive labels, emissions may be detected directly, e.g. with particle counters or indirectly, e.g. with scintillation cocktails and counters. In a specific embodiment, the bottom of the filtration tube is heat-melt sealed by contacting the tube-bottom with a hot plate, scintillation cocktail is added to the reservoir, and the radiolabel present in the tube counted.

detection

The disclosed methods are particularly suited to automated high throughput drag screening. In a preferred embodiment, the individual sample incubation volumes are less than about 500 ul, preferably less than about 250 ul, more preferably less than about 100 ul. Such small sample volumes minimize the use of often scarce candidate agent, expensive enzymes, and hazardous radioactive waste. Furthermore, the methods provide for automation, especially computerized automation. Accordingly, the method steps are preferably performed by a computer-controlled electromechanical robot. While individual steps may be

separately automated, a preferred embodiment provides a single computer-controlled multifunction robot with a single arm axially rotating to and from a plurality of work stations performing the mixture forming, incubating and separating steps. The computer is loaded with software which provides the instructions which direct the arm and work station operations and provides input (e.g. keyboard and/or mouse) and display (e.g. monitor) means for operator interfacing.

In a particular embodiment, the robotic arm is equipped with a general purpose retrieving hand and a pipetting hand. The pipetting hand equipped with a multichannel pipettor retrieves and transfers measured aliquots of each an assay buffer, a solution comprising one or more candidate agents, a solution comprising a labeled oligonucleotide hybridized to a plasmid or viral template, and a solution comprising the targeted helicase activity into each designated filtration well of a microtiter plate, wherein each well comprises an upper reservoir portion with a bottom made of a solvent-permeablizing membrane. The general purpose hand then transfers each microtiter plate to an incubator. After a first incubation period for a time and at a temperature to permit assay-detectable unwinding (e.g. 0.5 to 1.5 hours at 38° C.), the general purpose hand transfers each plate to a microbead dispensing station (e.g. a Multidrop system) which deposits in each designated well a measured aliquot of a slurry of GLASSFOG® (BIO 101, tel: (800)424-6101) microbeads and a solution of an organic solvent (e.g. methanol) at a concentration sufficient to permeablize the filter to water (e.g. about 12.5% v/v, final concentration), yet retain the beads. After a second incubation period for a time and at a temperature to permit assay-detectable immobilization (e.g. 0.5 to 1.5 hours at room temperature), the general purpose hand transfers each plate to a vacuum diaphragm where the substantially all of the liquid phase is simultaneously filtered from each well. A measured aiquot of wash solution is then added and then vacuum filtered through each well until background counts are reduced to an assay-acceptable level. Optionally, the bottom of each plate may be blotted onto an absorbent membrane after one or more filtration steps to remove residual liquid from the bottom of each well. The bottom of the drip director of each well is then sealed (e.g. heat melt sealed) and a measured aliquot of scintillation cocktail added to each well. Thereafter, the mount of label retained in each designated well is quantified.

Assays for helicase activity per se are carried out substantially as described above except for the omission of the candidate agent. Furthermore, candidate helicase activity samples are compared with one or more known helicase activities, preferably a panel of defined activities. The candidate helicase samples are typically cellular or nuclear extracts.

In a preferred embodiment, the panel of control helicases comprises a range of different activities to maximize the likelihood of encompassing an activity functionally similar, in terms of the subject assay, to that sought to be detected in the sample.

The following examples are offered by way of illustration and not by way of limitation.

EXAMPLES

Example 1.

Preparation of Labeled M13 Substrate

Annealing Reaction	
500 ul	M13mp18 ssDNA (500 ug, 250 pmoles total)
6.4 ul	M13-complementary 22-mer oligonucleotide primer (1 ug/ul, 1000 pmoles total)
140 ul	10X Annealing Buffer (500 mM NaCl, 200 mM $MgCl_2$ and 400 mM TRIS™-Cl, pH 7.5)
53.6 ul	ddH_2O
700 ul	Total
70 C., 10 min., then 55 C., 1 hr. Store at −20 C. until further use.	
Labeling Reection	
175 ul	Annealing reaction (125 ug total)
3.0 ul	0.5M DTT (5 mM final)
100 ul	^{33}P-dATP (2000 Ci/mmol, 1 mCi total, 500 pmol total)
24 ul	Klenow fragment (5 Units/ul)
302 ul	Total
Mix by gentle vortex, then buzz in microfuge to bring to bottom. Incubate at 25° C. (room temperature) for 45 min.	

QUICKSPIN™ Column Preparation

At 30 min. into the labeling reaction, remove 2 Large Scale QUICKSPIN™ columns (3.0 ml bed volume). Invert several times to mix resin, then remove top cap, followed by bottom cap. Place column in collection tube and spin at 2,000 rpm for 4 min. Remove supernatant from collection tube and spin again at 2,000 rpm for 4 min. Replace the collection tube with an EPPENDORF™ tube and store upright until ready to use.

Removal of Klenow and Unicorporated Nucleotide

Add 300 ul Phenol/Chloroform/Isoamyl alcohol (50:49:1). Vortex briefly, then spin in microfuge 2 minutes. Carefully remove as much of the upper aqueous phase as possible without disturbing interface. Transfer aqueous phase to QUICKSPIN™ column that has been "spun dry." Spin at 2,000 rpm for 6 minutes. Collect supernatant (take note of volume) and transfer to another QUICKSPIN™ column and spin at 2,000 rpm for 6 minutes. Remove supernatant to sterile 1.5 ml EPPENDORF™ tube. Take accurate volume measurement.

Example 2.

HSV Helicase/Primase Manual Assay Protocol

Final Conditions

Reaction is done on a polypropylene 96 well plate. The total reaction volume is 100 μl. Add assay buffer

20 mM HEPES™ (pH 7.6) 4 mM $MgCl_2$ 4 mM ATP 100 μg/ml BSA 5% glycerol 2 mM DTT

Add 10% DMSO. Add UL5/52 Helicase. Add ^{33}P dATP labeled oligo annealed on the M13 template. Incubate for 1 hr at 25° C. Add 2× capture buffer containing silica beads.

25% MeOH 3.0M NaI 0.3% RIP-40™ 10% GLASSFOG® beads

Incubate for 30 min. at 25° C. Transfer to GF/C Unifilter hydrophilic plate using cell harvester Wash five times with wash buffer

50% EtOH 0.2% RIP-40™ 50 mM NaCl 1 mM EDTA

Count

Seal plates using hot plate. Add 160 ul of scintillation cocktail. Seal bottom and top with Packard plate sealer. Count in Topcount scintillation counter.

Example 3.

HSV OBP Automated Helicase Assay Protocol

1. 1× Helicase Assay Buffer from 5× Stock Assay Buffer 5× stock Helicase assay buffer

Concentration	100 ml
20 mM CHES™	10 ml 1M (pH 9, KOH)(Sigma cat # C2885)
8 mM MgCl	4 ml of 1M
500 ug/ml BSA	250 mg
3% Glycerol	15 ml
	Add ddH2O to 100 ml

To make 500 ml of 1× helicase assay buffer add fresh daily:

Concentration	
5x helicase buffer	100 ml 5x buffer
2.5 mM DTT (to final conc.)	250 ul of 5M
8 mM ATP (to final conc.)	2.2 g
	Add ddH_2O to 500 ml

pH the 1x buffer to pH 9.0 using 10 N NaOH

2. 5× Substrate/Enzyme Buffer (SEB) Add Fresh Daily

Concentration	100 ml
20 mM CHES™	10 ml 1M
500 ug/ml BSA	250 mg
3% Glycerol	15 ml
100 mM KCl	16.5 ml 3M

To make 1× add fresh daily:

	100 ml
5 x SEB	20 ml
2.5 mM DTT	50 ul 5M
	Add ddH_2O to 100 ml

pH the 1x buffer to pH 9.0 using 10 N NaOH

3. Wash Buffer

Concentration	
50% EtOH	2 L (RICCA™ from Baxter cat. # 65915)
1 mM EDTA	8 ml 0.5M
0.2% np-40™	8 ml (Calbiochem cat. # 492015)
50 mM NaCl	40 ml 5M
	Add 4 L with ddH2O

4. GLASSFOG® Silica Beads

Concentration	1 L
25% MeOH	250 ml (Fisher cat. # A452-4)
3M NaI	500 ml 6M (BIO 101 cat. # 1001-801)
0.3% np-40™	3 ml
10% BIOBEADS	100 ml (BIO 101 cat. # 1005-904)
	Bring up to 1 L with dd H2O

5. M13/Enzyme Mixture

M13 fixed at 2.7 ng/well Stock M13 is 380 ng/ul (Apr. 26, 1995) in TE OBP fixed at 4.0 ng/well Stock OBP is 1000 ng/ul (Apr. 17, 1995) in storage buffer (20 mM Hepes, pH7.9, 1M

KCl, 15% Glycerol, 1 mM EDTA, 1 mM DTT, 1 mM NaMetabisulfate, 1 mM AEBSF (4-2-aminoethyl 7-benzenesulfyl fluoride hydorchloride

Preparation of M13 Solution

Volume needed for M13/OBP mixture (V)=# of plates×1 ml+12 ml Amount of M13 stock to be dissolved in V ml of SEB, (A) Multiply .27 ng/ul by the total volume needed and divide by stock concentration of M13.

Ex. V = 48 X 1 ml + 12 ml = 60 ml
Stock M13 conc. = 380 ng/ul
A = (.27 × 60,000 ul)/380 =
42.6 ul of stock M13 into 60 ml of SEB

Preparation of OBP Solution

Volume needed for M13/OBP mixture (V)=# of plates×1 ml+12 ml Amount of OBP stock to be dissolved in V ml of SEB, (B) Multiply 0.4 ng/ul by the total volume needed and divide by stock concentration of OBP.

Ex. V = 48 X 1 ml + 12 ml = 60 ml
Stock OBP conc. = 1000 ng/ul
B = (.45 × 60,000 ul)/1000
= 27 ul of stock OBP into 60 ml of SEB

6. Robotic Steps on ZYMARK™ Workstation T2

Add 15 ul 3M KCl to first column of hydrophobic GF/C plates (control for total binding of M13 template under conditions which inactivate OBP ie. high salt).

Add 80 ul of helicase buffer/well. Add 10 ul of compound/well. Add 10 ul of labeled substrate and enzyme in SEB/well. Shake 10 min. Place plates in incubator for 1 hr. at 38° C. Remove plates from incubator and add 100 ul of GLASS-FOG® beads. Sit for 30 minutes at 25° C. Wash plates with wash buffer.

7. Sealing and Counting of Plates

Seal plates using hot plate. Add 160 ul of scintillation cocktail. Seal bottom and top with PACKARD plate sealer. Count in TOPCOUNT scintillation counter.

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference. Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

What is claimed is:

1. A method of identifying an agent that modulates the activity of a helicase comprising the following steps in consecutive order:

a) forming a mixture of a labeled first nucleic acid hybridized to a complementary unlabeled second nucleic acid, a helicase, a nucleoside triphosphate, and a candidate agent, wherein said first and second nucleic acids are both DNA or are both RNA;

b) incubating said mixture in a reservoir under conditions wherein said helicase would unhybridize said first and second nucleic acids in the absence of said agent;

c) immobilizing said second nucleic acid on a solid substrate by direct noncovalent binding, wherein said second nucleic acid has a greater affinity for said solid substrate than does the first nucleic acid;

d) separating any unhybridized labeled first nucleic acids to obtain isolated, immobilized second nucleic acids;

e) measuring the amount of said label retained on the immobilized second nucleic acid, wherein the amount of said label is indicative of the modulation of helicase activity by said agent.

2. The method of claim 1, wherein said solid substrate is a micro-bead and said greater affinity results from said second nucleic acid being of greater length than said first nucleic acid.

3. The method of claim 1, wherein said second nucleic acid comprises a ligand, wherein said greater affinity results from said ligand having a greater affinity for said solid substrate than does said first nucleic acid.

4. The method of claim 3, wherein said solid substrate is coated with avidin, said ligand is biotin and said label is other than biotin.

5. The method of claim 3, wherein said solid substrate is coated with a digoxigenin-specific antibody, said ligand is digoxigenin and said label is other than digoxigenin.

6. The method of claim 1, wherein said label is a radiolabel.

7. The method of claim 1, wherein said label is a fluorescent label.

8. The method of claim 3, wherein said label is biotin and said ligand is other than biotin.

9. The method of claim 3, wherein said label is digoxigenin and said ligand is other than digoxigenin.

10. The method of claim 1, wherein said separating step comprises filtering said solid substrate.

11. The method of claim 1, wherein said separating step is performed in a tube having a fluid passage comprising a reservoir portion and a evacuation portion a filter extending transversely across said passage and separating said reservoir portion from said evacuation portion, said filter being changeable from water-impermeable to water-permeable having a maximam pore size sufficiently large to freely pass said any unhybridized first nucleic acid and sufficiently small to retain said solid substrate.

12. The method of claim 10, wherein said filter comprises a first membrane being changeable from water-impermeable to water-permeable by contacting said first membrane with an effective concentration of an organic solvent and a second membrane being water-permeable and having a maximam pore size less than about 200 um diameter.

13. The method of claim 1, wherein said helicase is a pathogenic helicase.

14. The method of claim 1, wherein said hellcase is a pathogenic fungal helicase.

15. The method of claim 1, wherein said helicase is a pathogenic human helicase.

16. The method of claim 1, wherein said helicase is a pathogenic bacterial helicase.

17. The method of claim 1, wherein said helicase is a pathogenic viral helicase.

* * * * *

US006303315B1

(12) **United States Patent**
Skouv

(10) **Patent No.: US 6,303,315 B1**
(45) **Date of Patent: Oct. 16, 2001**

(54) **ONE STEP SAMPLE PREPARATION AND DETECTION OF NUCLEIC ACIDS IN COMPLEX BIOLOGICAL SAMPLES**

(75) Inventor: **Jan Skouv**, Espergærde (DK)

(73) Assignee: **Exiqon A/S**, Vedbaek (DK)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **09/528,271**

(22) Filed: **Mar. 18, 2000**

Related U.S. Application Data

(60) Provisional application No. 60/127,356, filed on Apr. 1, 1999.

(30) **Foreign Application Priority Data**

Mar. 18, 1999 (DK) 1999 00384

(51) **Int. Cl.**[7] **C12Q 1/68**

(52) **U.S. Cl.** **435/6**; 435/5; 435/6; 435/91.1; 435/91.2; 536/23.1; 536/24.3; 536/32; 536/33; 422/68.1

(58) **Field of Search** 435/6, 5, 91.2; 536/23.1, 24.3, 32, 33; 422/68.1

(56) **References Cited**

U.S. PATENT DOCUMENTS

5,821,060	10/1998	Arlinghaus et al.	435/6
5,942,391 *	6/2000	Zhang et al.	435/6
6,077,673 *	6/2000	Zhang et al.	435/6

FOREIGN PATENT DOCUMENTS

0538194 B1	4/1999	(EP).
WO 92/20703	11/1992	(WO).
WO98/22489	5/1998	(WO).
WO98/39352	9/1998	(WO).
WO99/14226	3/1999	(WO).

OTHER PUBLICATIONS

Tarkoy et al., *Helv. Chim. Acta,* 76:481 (1993).
Tarkoy et al., *Angew. Chem. Int. Ed. Engl.,* 32:1432 (1993).
Egli et al., *J. Am. Chem. Soc.,* 115:5855 (1993).
Tarkoy et al., *Helv. Chim. Acta,* 77:716 (1994).
Bolli et al., *Angew. Chem. Int. Ed. Engl.,* 34:694 (1995).
Bolli et al., *Helv. Chim. Acta,* 78:2077 (1995).
Litten et al., *Bioorg. Med. Chem. Lett.,* 5:1231 (1995).
Litten et al., *Helv. Chim. Acta,* 79:1129 (1996).
Bolli et al., *Chem. Biol.,* 3:197 (1996).
Bolli et al., *Nucleic Acids. Res.,* 24:4660 (1996).
K.H. Altmann et al., *Tetrahedron Lett.,* 35:2331 (1994).
K. H. Altmann et al., *Tetrahedron Lett.,* 35:7625 (1994).
Marquez et al., *J. Med. Chem.,* 39:3739 (1996).
Ezzitouni et al., *J. Chem. Soc., Perkin Trans.,* 1:1073 (1997).
Jones et al., *J. Am. Chem. Soc.,* 115:9816 (1993).
Wang et al., *Bioorg. Med. Chem. Lett.,* 7:229 (1997).
Yannopoulus et al., *Synlett,* 378 (1997).
CHIMA, 36[th] Congress, organized by the Swiss Chemical Society, Poster No. SB–B4: Steffens, R. and Leumann Ch. Tricyclo–DNA: synthesis, enzymatic stability, and pairing properties.
Nielsen, Master Thesis (Odense University, Denmark), p. 67–71 (1995).
Youssefyeh et al., *J. Org. Chem.,* 44:1301 (1979).
Jones et al., *J. Org. Chem.,* 44:1309 (1979).
Yang et al., *Tetrahedron Lett.,* 33:37 (1992).
Thrane et al., *Tetrahedron,* 51:10389 (1995).
Nielsen et al., *Bioorg. Med. Chem.,* 3:1493 (1995).
Freier et al., *Nucleic Acid Research,* 25:4429–4443 (1997).
Haly et al., *Synlett,* 687–689 (1996).
Zou et al., *Tetrahedron Lett.,* 37:941–944 (1996).
Herdewijn., *Liebigs Ann.,* 1337–1348 (1996).
Obika et al., *Tetrahedron Lett.,* 39:5401–5404 (1998).
Obika et al., *Tetrahedron Lett.,* 38:8735–8738 (1997).
7[th] Antisense Symposium, Nov. 21–22, 1997. Poster No. 32 and 33: Obika, D.N.; Morio, K. and Imanishi, T. Synthesis and properties of oligonucleotides containing novel bicyclic nucleosides with a fixed N–form sugar puckering.
CHIMA, 36[th] IUPAC Congress, organized by the Swiss Chemical Society. Poster No. SB–B12: Egtger, A. and Leumann Ch. Designe, synthesis and properties of bicyclo [3.2.1]–amino nucleic acids.
CHIMA, 36[th] IUPAC Congress, organized by the Swiss Chemical Society. Poster No. SB–B5: Epple, C. Ch., Pompizi, I. and Leumann Ch. Bicyclo [3.2.1]–DNA: an oligonucleotide analogue with a conformationally preorganized Phosphodiester backbone and a flexible sugar–base linkage.
Sep. 6–10, 1998: 13[th] International Round Table—Nucleoside, Nucleotides and their Biological Applications, Montpeilleir: Oral Communication 1: Wang, G. and Gunic, E. "Conformationally Locked Nucleoside Analogs. Synthesis of 2'–Deoxy–2'–C, 4'–C–Bridged Bicyclic Nucleoside".

(List continued on next page.)

Primary Examiner—W. Gary Jones
Assistant Examiner—Janell E. Taylor
(74) *Attorney, Agent, or Firm*—Peter F. Corless; Edwards & Angell, LLP

(57) **ABSTRACT**

A method for simultaneous release and detection of nucleic acids from complex biological samples is described. The invention relates to the combined use of lysis buffers containing strong chaotropic agents such as guanidine thiocyanate to facilitate cell lysis and release of cellular nucleic acids and to the use of a novel type of bicyclic nucleotide analogues, locked nucleic acid (LNA) to detect specific nucleic acids released during lysis by nucleic acid hybridisation. In particular methods are described for the covalent attachment of the catching LNA-oligo. Novel methods for sample preparation of e.g. polyadenylated mRNA species are also presented. The invention further addresses reagents for performing the methods as well as reagents and applications of the method.

65 Claims, 11 Drawing Sheets

OTHER PUBLICATIONS

Sep. 6–10, 1998: 13th International Round Table—Nucleoside, Nucleotides and their Biological Applications, Montpeilleir: Poster No. 288: Meldgaard, M. et al., "LNA (Locked Nucleic Acids): Synthesis and Thermal Denaturation Studies".

Sep. 6–10, 1998: 13th International Round Table—Nucleoside, Nucleotides and their Biological Applications, Montpeilleir: Oral Communication 1: Wang, G. and Gunic, E. "Conformationally Locked Nucleoside Analogs. Synthesis of 2'-Deoxy-2'-C, 4'-C-Bridged Bicyclic Nucleoside".

Sep. 6–10, 1998: 13th International Round Table—Nucleoside, Nucleotides and their Biological Applications, Montpeilleir: Poster No. 67: Nielsen, P. and Wengel, J. "A New Convergent Synthetic Approach Towards α-and β-LNA (Locked Nucleic Acids)".

Oct. 8, 1998: Antisense 98, Targeting the Moleculari Basis of Disease: Poster No. 24: Havsteen, M. et al., "LNA (Locked Nucleic Acids): A new Class of High Affinity Nucleic Acids With Prime Potential as Antisense and Antigene Agents".

Jan. 21, 1998: National Seminar on Perspectives in Interfacial Areas of Chemistry and Biology, Delhi University: Wengel, J. "LNA (Locked Nucleic Acids): Synthesis and High Affinity Nucleic Acid Recognition—Stop the Twisting."

Mar. 27, 1998: Workshop of Young European Bioorganic Chemists, Munchen: Wengel, J. "LNA (Locked Nucleic Acids): Synthesis and High Affinity Nucleic Acid Recognition—Stop the Twisting."

Aug. 20, 1988: Årsmødet for Center for Medicinsk Biotechnologi, KVL: Wengel, J. "LNA (Locked Nucleic Acids)"ations, Montpeilleir: Oral Communication 1: Wang, G. and Gunic, E. "Conformationally Locked Nucleoside Analogs. Synthesis of 2'-Deoxy-2'-C, 4'-C-Bridged Bycyclic Nucleoside".

Sep. 7, 1998: 13th International Round Table—Nucleoside, Nucleotides and their Biological Applications, Montpeilleir: Oral Communication 2: Wengel, J. "LNA (Locked Nucleic Acids)".

Sep. 8, 1998: Meeting in Lund, Sweden: Jakobsen, M. H. "LNA (Locked Nucleic Acids): A new Class of High Affinity Nucleic Acids With Prime Potential as Antisense and Antigene Agents".

Nielsen et al., *J. Chem. Soc., Perkin Trans.,* 1:3423–3433 (1997).

Nielsen et al., *Chem. Commun.,* 9:825–826 (1997).

Singh et al., *Chem. Commun.,* 9:455–456 (1998).

Koshkin et al., *Tetrahedron,* 54:360733630 (1998).

Koshkin et al., *Tetrahedron Lett.,* 39:4381–8384 (1998).

Singh et al., *Chem. Commun.,* 1237–1248 (1998).

Singh et al., *J. Org. Chem.,* 63:6078–6079 (1998).

Christensen et al., *J. Am. Chem. Soc.,* 120:5458–5463 (1998).

Koshkin et al., *J. Org. Chem.,* 63:2778–2781 (1998).

Kumar et al., *Bioorg. Med. Chem. Lett.,* 8:2219–2222 (1998).

Wengel et al., *Acc. Chem. Res.,* 32:301–310 (1999).

Koshkin et al., *J. Am. Chem. Soc.,* 120:13252–13253 (1998).

Singh et al., *J. Org. Chem.,* 10035–10039 (1998).

Nielsen et al., *Chem. Commun.,* 2645–2646 (1998).

Wengel et al., *Nucleosides Nucleoties,* 28:1365–1370 (1999).

Nielsen et al., *Nucleosides Nucleotides,* 18:701–702 (1999).

Kærno et al., *Chem. Commun.,* 657–658 (1999).

Rajwanshi et al., *J. Chem. Soc., Perkin Trans.,* 1:1407–1414 (1999).

Raunkjær et al., *J. Chem. Soc. Perkin Trans.,* 1:2543–2551 (1999).

Rajwanshi et al., *Chem. Commun.,* 1395–1396 (1999).

Pfundheller et al., *Nucleosides Nucleotides,* 18:2017–2030 (1999).

Rajwanshi et al., *Chem. Commun.,* 2073–2074 (1999).

Nielsen et al., *J. Biomol. Struc. Dyn.,* 17:175–191 (1999).

Nielsen et al., *Bioconjugate Chem.,* 11:228–238 (2000).

Rajwanshi et al., *Angewandte Chemie,* 39:1656–1659 (2000).

Minasov et al., *Biochemistry,* 39:3525 (2000).

Wahlesttedt et al., *Proc. Natl. Acad. Sci. USA,* 97:5633–5638 (2000).

Obika et al., *Tetrahedron Lett.,* 40:6465–6468 (1999).

Obika et al., *Tetrahedron Lett.,* 41:215–219 (1999).

Obika et al., *J. Chem. Soc., Chem. Commun.,* 2423–2424 (1999).

Wang et al., *Bioorg. Med. Chem. Lett.,* 9:1147–1150 (1999).

Obika et al., *Tetrahedron Lett.,* 41:221–224 (1999).

Obika et al., *Bioorg. Med. Chem. Lett.,* 9:515–518 (1999).

Obika et al., *Tetrahedron Lett.,* 39:5401–5405 (1998).

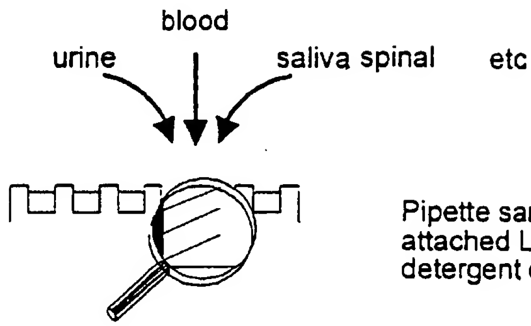
Imanishi et al., *J. Synth. Org. Chem.,* 57:959–980 (1999).

Chemical Abstracts, vol. 70, No. 1, Abstract No. 3737B (1969).

Monatsch. Chem., 99(5):2111–2120 (1968).

* cited by examiner

Illustration of principle

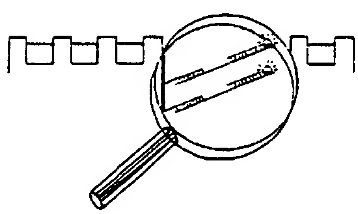


Pipette sample into tube or well with covalently attached LNA catching oligos, lysis buffer, detergent detection probe (LNA) etc.

FIG. 1A

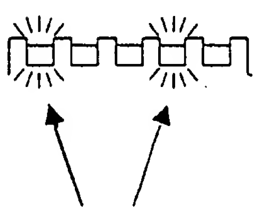
cells lyse, proteins denaturate and nucleic acids are released into solution.

FIG. 1B



due to the high Tm of LNA's nucleic acid are caught by the catching LNA oligo and hybridize with the detection LNA.

FIG. 1C



Incubate, wash and add, developing mixture.

develop and read the result of test.

positive samples

FIG. 1D

Fig. 1-1

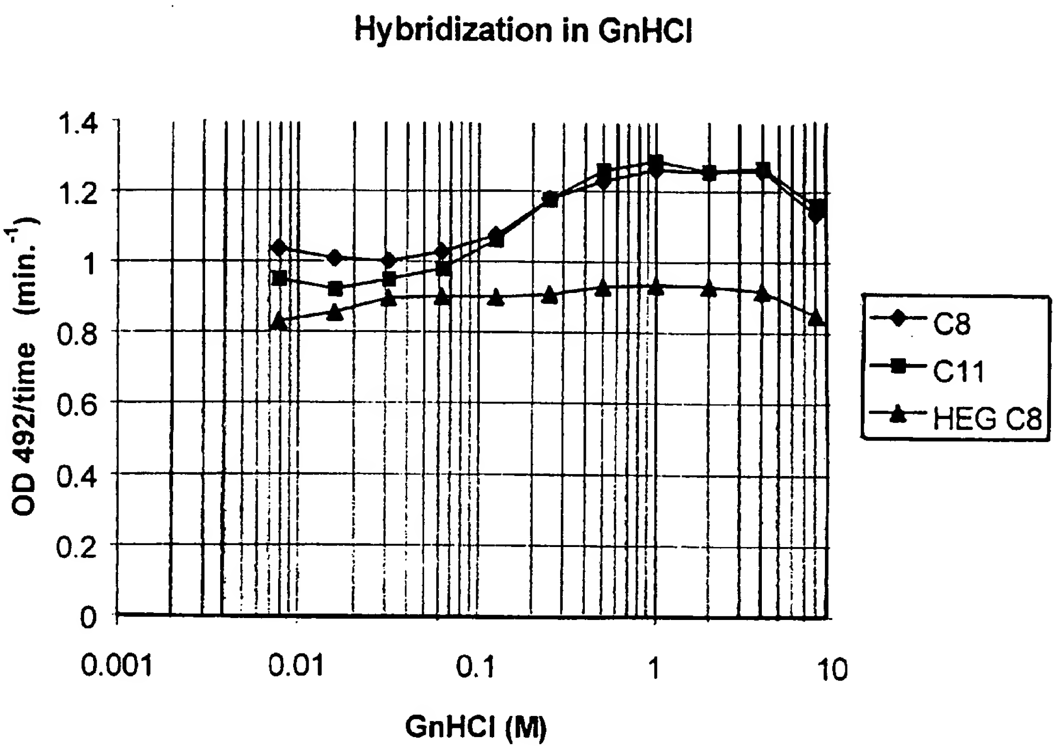
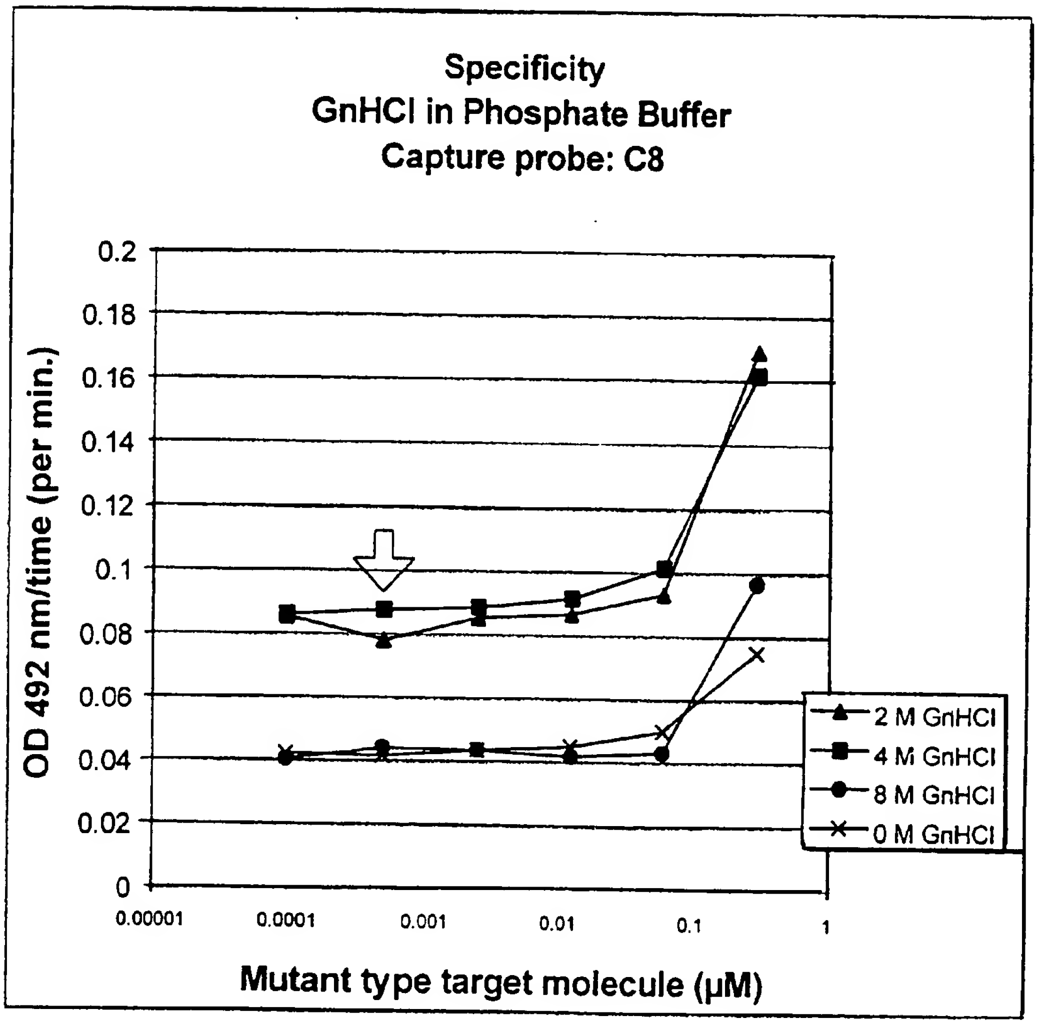

Hybridization in GnHCl
OD 492/time (min.$^{-1}$)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0.001
0.01
0.1
1
10
GnHCl (M)
C8
C11
HEG C8

Fig. 2-1



Specificity
GnHCl in Phosphate Buffer
Capture probe: C8
OD 492 nm/time (per min.)
0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
0.00001
0.0001
0.001
0.01
0.1
1
Mutant type target molecule (μM)
2 M GnHCl
4 M GnHCl
8 M GnHCl
0 M GnHCl

Fig. 2-2

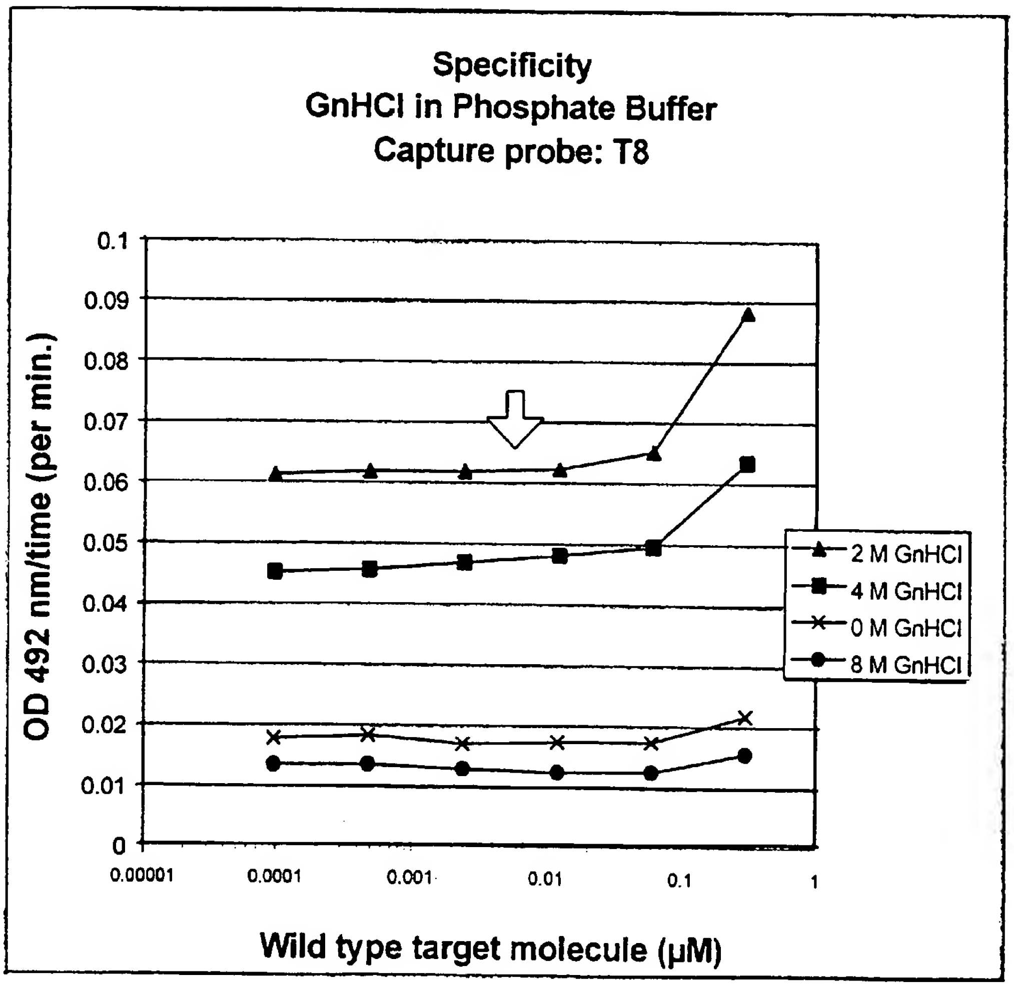
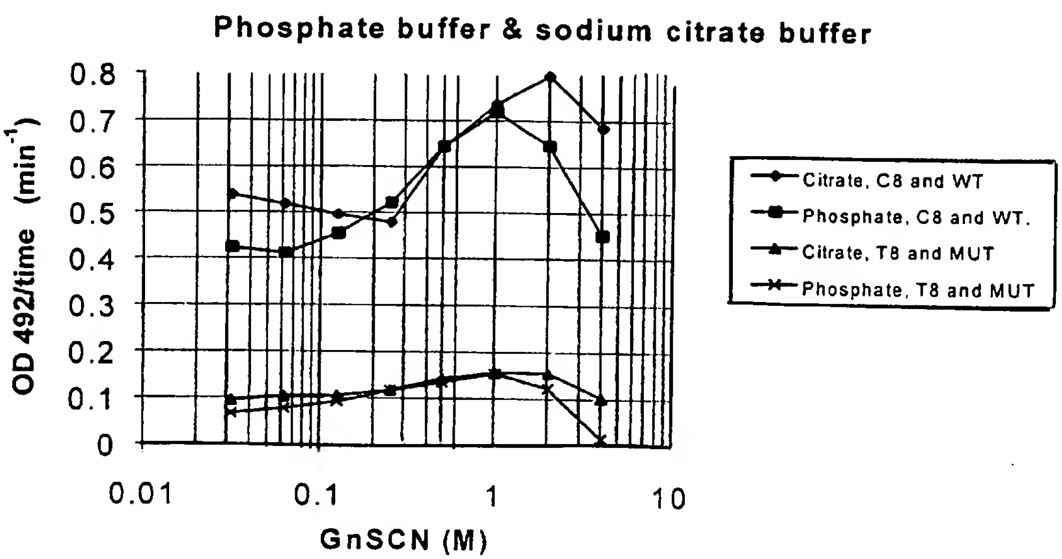

Specificity
GnHCl in Phosphate Buffer
Capture probe: T8
OD 492 nm/time (per min.)
0.1
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
0.00001
0.0001
0.001
0.01
0.1
1
Wild type target molecule (μM)
2 M GnHCl
4 M GnHCl
0 M GnHCl
8 M GnHCl

Fig. 3-1



Phosphate buffer & sodium citrate buffer
OD 492/time (min-1)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0.01
0.1
1
10
GnSCN (M)
Citrate, C8 and WT
Phosphate, C8 and WT.
Citrate, T8 and MUT
Phosphate, T8 and MUT

Fig. 4-1

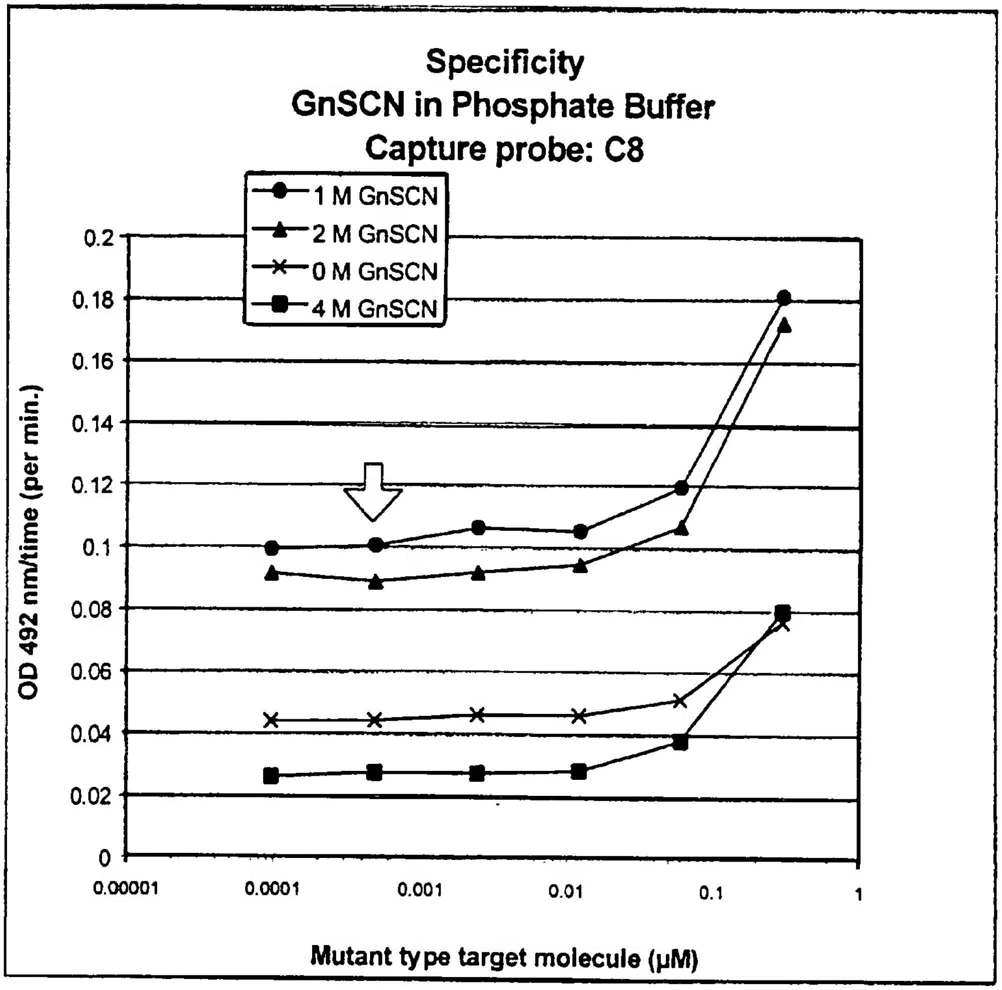

Specificity
GnSCN in Phosphate Buffer
Capture probe: C8
1 M GnSCN
2 M GnSCN
0 M GnSCN
4 M GnSCN
OD 492 nm/time (per min.)
0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
0.00001
0.0001
0.001
0.01
0.1
1
Mutant type target molecule (μM)

Fig. 4-2

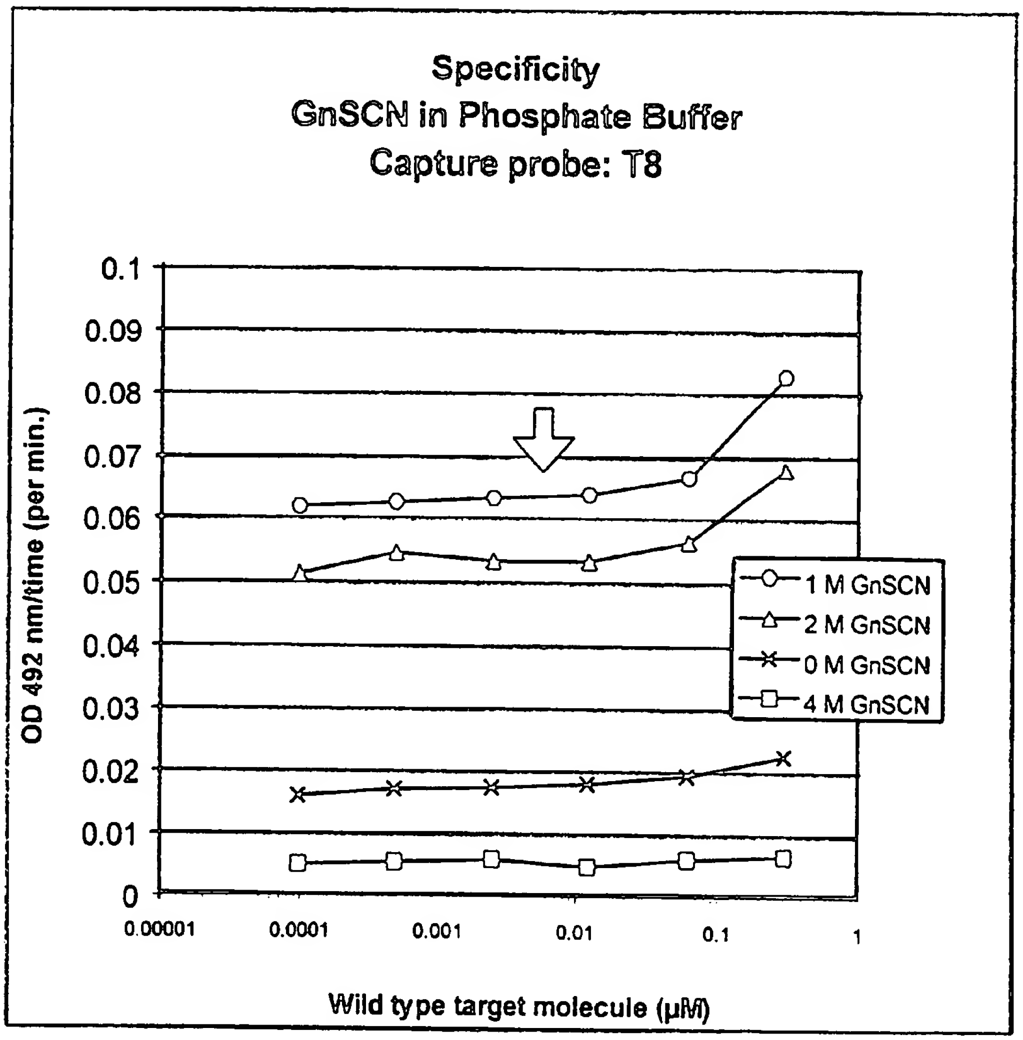

Specificity
GnSCN in Phosphate Buffer
Capture probe: T8
0.1
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
OD 492 nm/time (per min.)
0.00001
0.0001
0.001
0.01
0.1
1
Wild type target molecule (μM)
1 M GnSCN
2 M GnSCN
0 M GnSCN
4 M GnSCN

Fig. 4-3

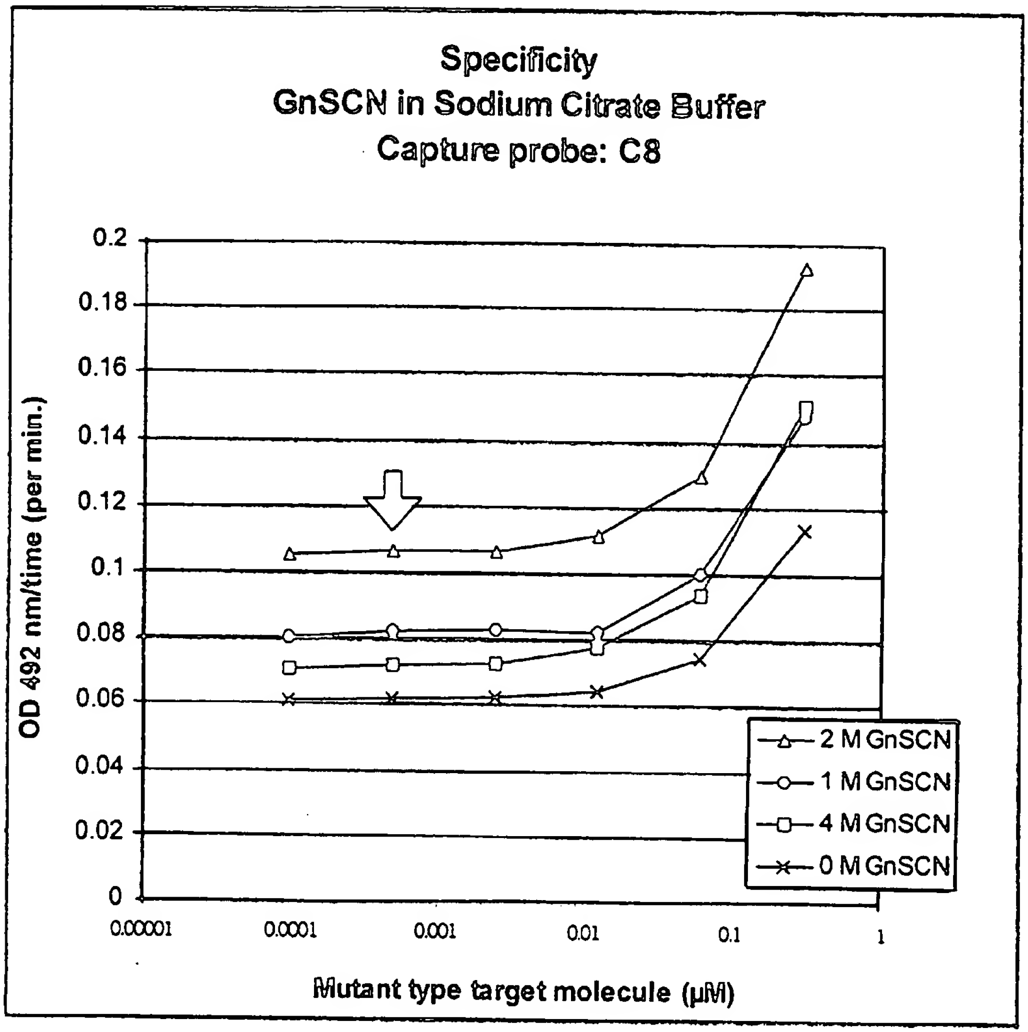

Specificity
GnSCN in Sodium Citrate Buffer
Capture probe: C8
0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
OD 492 nm/time (per min.)
0.00001
0.0001
0.001
0.01
0.1
1
Mutant type target molecule (μM)
2 M GnSCN
1 M GnSCN
4 M GnSCN
0 M GnSCN

Fig. 4-4

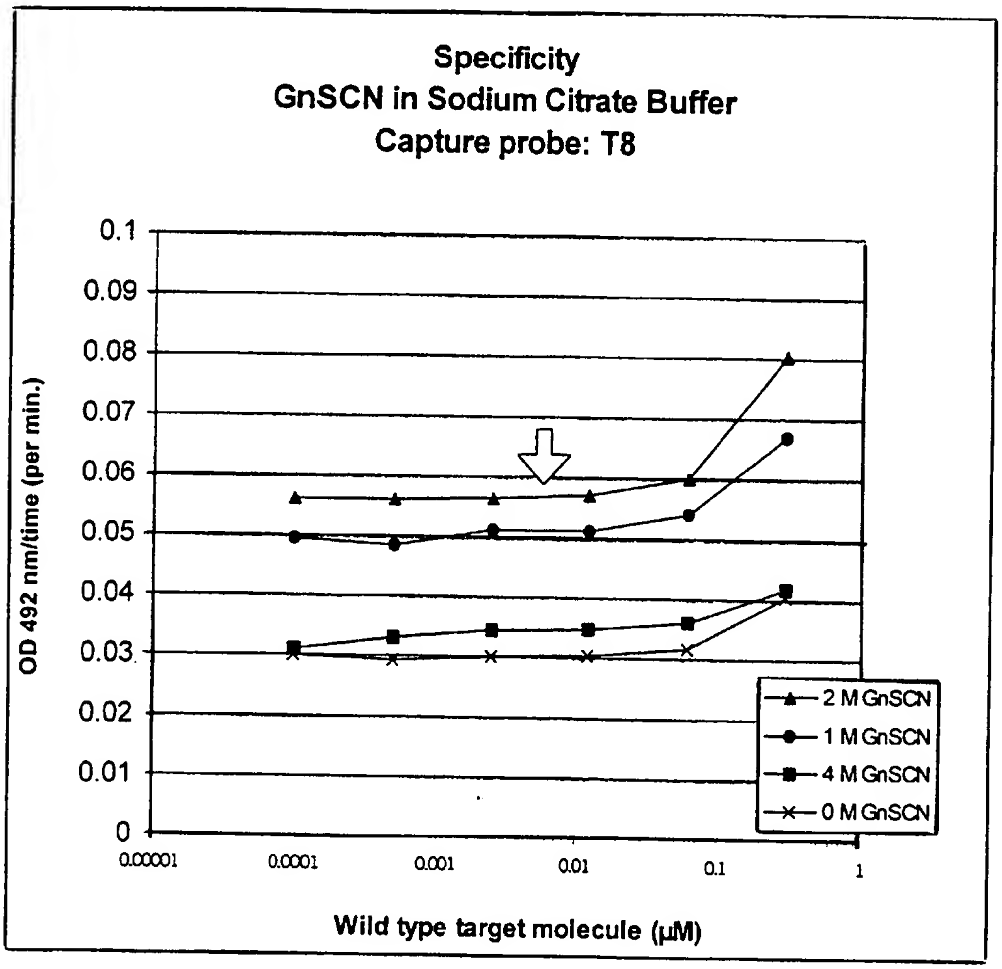

Specificity
GnSCN in Sodium Citrate Buffer
Capture probe: T8
OD 492 nm/time (per min.)
0.1
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
0.00001
0.0001
0.001
0.01
0.1
1
Wild type target molecule (μM)
2 M GnSCN
1 M GnSCN
4 M GnSCN
0 M GnSCN

Fig. 6-1

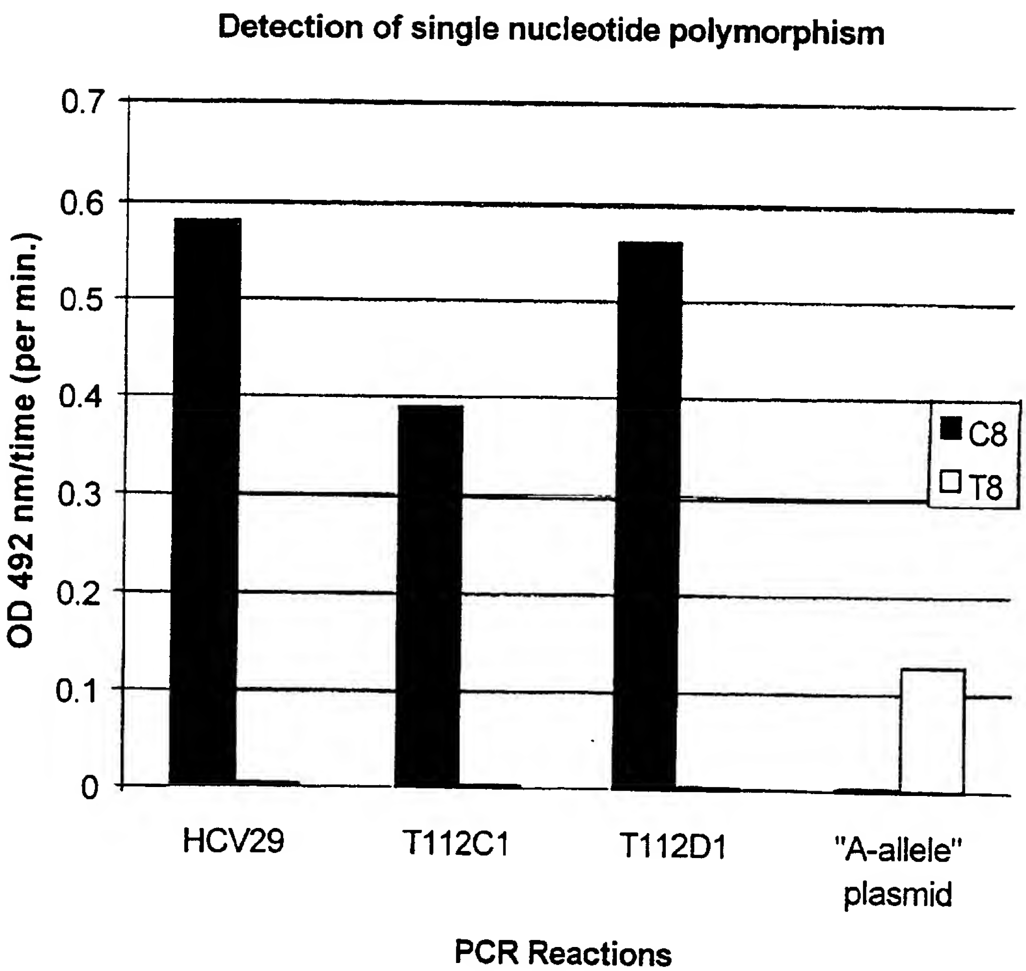
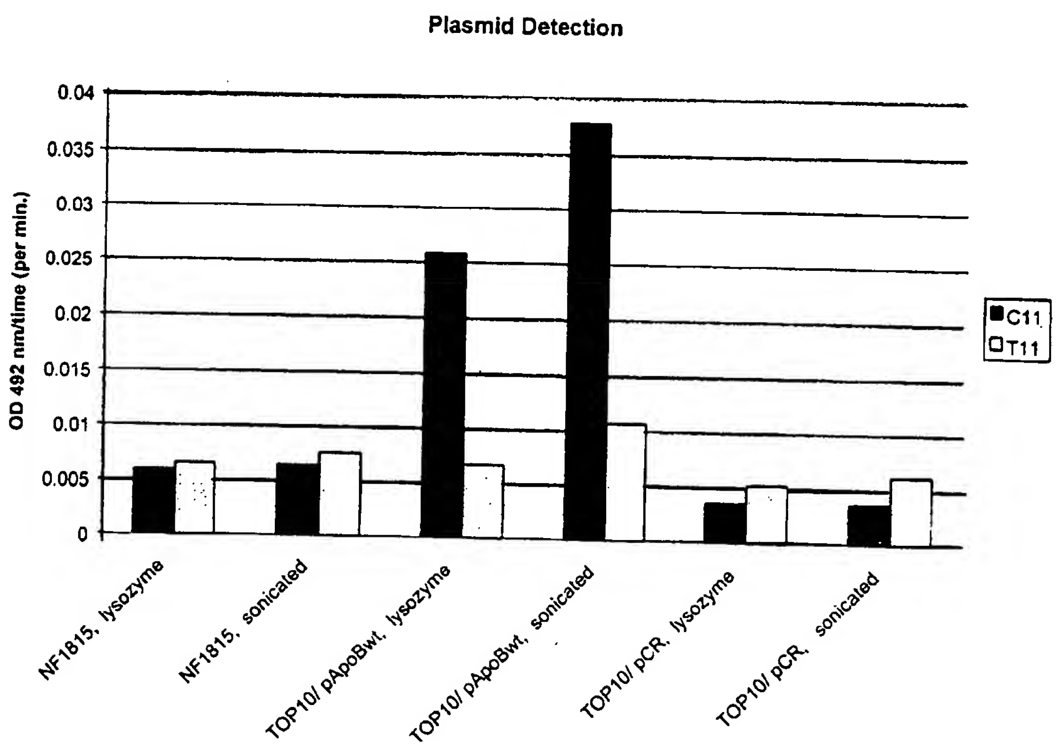

Detection of single nucleotide polymorphism
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
OD 492 nm/time (per min.)
C8
T8
HCV29
T112C1
T112D1
"A-allele" plasmid
PCR Reactions

Fig. 7-1


Plasmid Detection
OD 492 nm/time (per min.)
0.04
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
C11
T11
NF1815, lysozyme
NF1815, sonicated
TOP10/ pApoBwt, lysozyme
TOP10/ pApoBwt, sonicated
TOP10/ pCR, lysozyme
TOP10/ pCR, sonicated

ONE STEP SAMPLE PREPARATION AND DETECTION OF NUCLEIC ACIDS IN COMPLEX BIOLOGICAL SAMPLES

This application claims benefit to Provisional Application No. 60/127,356, filed Apr. 1, 1999.

BACKGROUND OF THE INVENTION

Brief Description of the Relevant Art

Organic solvents such as phenol and chloroform are traditionally used in techniques employed to isolate nucleic acid from prokaryotic and eukaryotic cells or from complex biological samples. Nucleic acid isolations typically begin with an enzymatic digest performed with proteases followed by cell lysis using ionic detergents and then extraction with phenol or a phenol/chloroform combination. The organic and aqueous phases are separated and nucleic acids which have partitioned into the aqueous phase are recovered by precipitation with alcohol. However, phenol or a phenol/chloroform mixture is corrosive to human skin and is considered as hazardous waste which must be carefully handled and properly discarded. Further, the extraction method is time consuming and laborious. Marmur, J. Mol. Biol., 3:208–218 (1961), describes the standard preparative procedure for extraction and purification of intact high molecular weight DNA from prokaryotic organisms using enzymatic treatment, addition of a detergent, and the use of an organic solvent such as phenol or phenol/chloroform. Chirgwin et al., Biochemistry, 18:5294–5299 (1979) described the isolation of intact RNA from tissues enriched in ribonuclease by homogenization in GnSCN and 2-mercaptoethanol followed by ethanol precipitation or by sedimentation through cesium chloride. Further developments of the methods are described by Ausubel et. al in Current Protocols in Molecular Biology, pub. John Wiley & Sons (1998).

Further, the use of chaotropic agents such as guanidine thiocyanate (GnSCN) is widely used to lyse and release nucleic acid from cells into solution, largely due to the fact that the chaotropic salts inhibit nucleases and proteases while at the same time facilitating the lysis of the cells.

Nucleic acid hybridisation is a known and documented method for identifying nucleic acids. Hybridization is based on base pairing of complementary nucleic acid strands. When single stranded nucleic acids are incubated in appropriate buffer solutions, complementary base sequences pair to form double stranded stable molecules. The presence or absence of such pairing may be detected by several different methods well known in the art.

In relation to the present invention a particular interesting technique was described by Dunn & Hassell in Cell, Vol.12, pages 23–36 (1977). Their assay is of the sandwich-type whereby a first hybridisation occurs between a "target" nucleic acid and a "capturing" nucleic acid probe which has been immobilized on a solid support. A second hybridisation then follows where a "signal" nucleic acid probe, typically labelled with a fluorophore, a radioactive isotope or an antigen determinant, hybridises to a different region of the immobilized target nucleic acid. The hybridisation of the signal probe may then be detected by, for example, fluorometry.

Ranki et al. in U.S. Pat. Nos. 4,486,539 and 4,563,419 and EP 0,079,139 describe sandwich-type assays which first require steps to render nucleic acids single stranded and then the single stranded nucleic acids are allowed to hybridise with a nucleic acid affixed to a solid carrier and with a

nucleic acid labelled with a radioisotope. Thus, the Ranki et al. assay requires the nucleic acid to be identified or targeted in the assay to be first rendered single stranded.

One approach to dissolving a biological sample in a chaotropic solution and performing molecular hybridisation directly upon the dissolved sample is described by Thompson and Gillespie, "Analytical Biochemistry," 163:281–291 (1987). See also WO 87/06621. Cox et al. have also described the use of GnSCN in methods for conducting nucleic acid hybridisation assays and for isolating nucleic acid from cells (EP-A-0-127-327).

Bresser, Doering and Gillespie, "DNA," 2:243–254 (1983), reported the use of NaI, and Manser and Gefter, Proc. Natl. Acad. Sci. USA, 81:2470–2474 (1984) reported the use of NaSCN to make DNA or mRNA in biological sources available for trapping and immobilization on nitrocellulose membranes in a state which was suitable for molecular hybridisation with DNA or RNA probes.

The use of LNA as capturing-probes and detecting oligos has not been investigated until now. Due to the extraordinary features of LNA, it is possible to obtain efficient hybridisation under conditions where DNA and RNA cannot form stable hybrids e.g. pure water or buffers containing detergents and high concentrations of strong chaotropic agents. Thus, it is possible to perform the steps of catching-hybridisation, detection-hybridisation and cell-lysis in one step. This offers a substantial simplification to previous published methods.

SUMMARY OF THE INVENTION

This invention relates to compositions and assay methods for the hybridisation and extraction of nucleic acids. In particular, this invention relates to compositions and methods to release nucleic acids from cells in complex biological samples or specimens while simultaneously hybridising complementary nucleic acids released during lysis. A crucial component in the invention is LNA which is a novel class of DNA analogues that possess some extraordinary features that make it a prime candidate for improving in vitro DNA diagnostics. The LNA monomers are bi-cyclic compounds structurally very similar to RNA-monomers, see formula I. LNA shares most of the chemical properties of DNA and RNA, it is water-soluble, can be separated by gel electrophoreses, ethanol precipitated etc. Furthermore, LNA oligonucleotides are conveniently synthesised by standard phosphoramidite chemistry. The phosphoramidite chemistry allows chimeric oligos containing both LNA and DNA (or RNA) monomers to be synthesized. Thus, mixed LNA/DNA oligos with a predefined melting temperature (T_m) can be prepared. The flexibility of the phosphoramidite synthesis approach furthermore facilitates the easy production of LNAs carrying all commercially available linkers, fluorophores and labelling-molecules available for this standard chemistry. Importantly, introduction of LNA monomers into either DNA or RNA oligos results in unprecedented high thermal stability of duplexes with complementary DNA or RNA while at the same time obeying the Watson-Crick base-pairing rules. In general the thermal stability of heteroduplexes is increased 3–8° C. per LNA-monomer in the duplex. To the best of our knowledge LNA has the highest affinity towards complementary DNA or RNA ever to be reported (Tetrahedron, 54, 3607–3630 (1998)). The thermal stability of LNA/DNA and LNA/RNA heteroduplexes is sufficiently stable to allow efficient hybridisation to occur even in the presence of chaotropic agents such as guanidine thiocyanate (GnSCN).

This invention relates to novel methods for the release of nucleic acids from cells in complex biological samples or specimens to prepare and make available the nucleic acid material present for a hybridisation assay. Novel methods for hybridisation of nucleic acids are also presented. In particular, methods are described for the hybridisation of nucleic acids from a sample suspected of containing a target nucleic acid of interest wherein the sample is combined with a buffer comprising at least one strong chaotropic agent which promotes cell lysis and release of the cellular nucleic acid while at the same time allowing hybridisation with LNA. The extent of hybridisation of the complementary nucleic acid to the target nucleic acid is then determined.

One advantage of these hybridisation methods is that hybridisation may be carried out in one easy step with all reagents pre-combined.

DETAILED DESCRIPTION

This invention relates to a novel method for detecting nucleic acids released from a lysed complex biological mixture containing nucleic acids. The methods of the present invention enable one to readily assay for a nucleic acid suspected of being present in cells, parts of cells or virus, i.e. target nucleic acid(s). Such methods include lysing the cells in a hybridisation medium comprising a strong chaotropic agent, contacting the lysate under hybridisation conditions with a locked nucleic acid (LNA) having a nucleotide sequence substantially complementary to a nucleotide sequence suspected to be present in the cells, and determining the extent of hybridisation.

The "target nucleic acid" means the nucleotide sequence of deoxyribonucleic acid (DNA), ribonucleic acid (RNA) (including ribosomal ribonucleic acid (rRNA), transfer RNA, (tRNA), small nuclear (snRNA), telomerase associated RNA, ribozymes etc.) whose presence is of interest and whose presence or absence is to be detected in the hybridisation assay. The nucleic acid sample of interest will be one which is suspected of containing a particular target nucleic acid, such as a particular gene, gene segment or RNA. Of particular interest is the detection of particular mRNAs which may be of eukaryotic, prokaryotic, Archae or viral origin. Importantly, the invention may assist in the diagnosis of various infectious diseases by assaying for particular sequences known to be associated with a particular microorganism. The target nucleic acid may be provided in a complex biological mixture of nucleic acid (RNA, DNA and/or rRNA) and non-nucleic acid. The target nucleic acids of primary preference are RNA molecules and, in particular rRNAs such as the 16S or 23S rRNA described in commonly assigned U.S. patent application Ser. No. 08/142,106, which is incorporated by reference herein. If target nucleic acids of choice are double stranded or otherwise have significant secondary and tertiary structure, they may need to be heated prior to hybridisation. In this case, heating may occur prior to or after the introduction of the nucleic acids into the hybridisation medium containing the capturing probe. It may also be desirable in some cases to extract the nucleic acids from the complex biological samples prior to the hybridisation assay to reduce background interference by any methods known in the art.

The hybridisation and extraction methods of the present invention may be applied to a complex biological mixture of nucleic acid (RNA and/or DNA) and non-nucleic acid. Such a complex biological mixture includes a wide range of eukaryotic and prokaryotic cells, including protoplasts; or other biological materials which may harbour target nucleic acids. The methods are thus applicable to tissue culture animal cells, animal cells (e.g., blood, serum, plasma, reticulocytes, lymphocytes, urine, bone marrow tissue, cerebrospinal fluid or any product prepared from blood or lymph) or any type of tissue biopsy (e.g. a muscle biopsy, a liver biopsy, a kidney biopsy, a bladder biopsy, a bone biopsy, a cartilage biopsy, a skin biopsy, a pancreas biopsy, a biopsy of the intestinal tract, a thymus biopsy, a mammae biopsy, an uterus biopsy, a testicular biopsy, an eye biopsy or a brain biopsy, homogenized in lysis buffer), plant cells or other cells sensitive to osmotic shock and cells of bacteria, yeasts, viruses, mycoplasmas, protozoa, rickettsia, fungi and other small microbial cells and the like. The assay and isolation procedures of the present invention are useful, for instance, for detecting non-pathogenic or pathogenic micro-organisms of interest. By detecting specific hybridisation between nucleotide probes of a known source and nucleic acids resident in the biological sample, the presence of the micro-organisms may be established.

Solutions containing high concentrations of guanidine, guanine thiocyanate or certain other chaotropic agents and detergents are capable of effectively lysing prokaryotic and eukaryotic cells while simultaneously allowing specific hybridisation of LNA probes to the released endogenous nucleic acid. The solutions need not contain any other component other than common buffers and detergents to promote lysis and solubilization of cells and nucleic acid hybridisation.

If extraction procedures are employed prior to hybridisation, organic solvents such as phenol and chloroform may be used in techniques employed to isolate nucleic acid. Traditionally, organic solvents, such as phenol or a phenol-chloroform combination are used to extract nucleic acid, using a phase separation (Ausubel et. al in Current Protocols in Molecular Biology, pub. John Wiley & Sons (1998)). These methods may be used effectively with the lysis solutions of the present invention; however, an advantage of the methods of the present invention is that tedious extraction methods are not necessary, thus improving the performance of high throughput assays. Preferably, the lysis buffer/hybridisation medium will contain standard buffers and detergents to promote lysis of cells while still allowing effective hybridisation of LNA probes. A buffer such as sodium citrate, Tris-HCl, PIPES or HEPES, preferably Tris-HCl at a concentration of about 0.05 to 0.5M can be used. The hybridisation medium will preferably also contain about 0.05 to 0.5% of an ionic or non-ionic detergent, such as sodium dodecylsulphate (SDS) or Sarkosyl (Sigma Chemical Co., St. Louis, Mo.) and between 1 and 10 mM EDTA. Other additives may also be included, such as volume exclusion agents which include a variety of polar water-soluble or swellable agents, such as anionic polyacrylate or polymethacrylate, and charged saccharidic polymers, such as dextran sulphate and the like. Specificity or the stringency of hybridisation may be controlled, for instance, by varying the concentration and type of chaotropic agent and the NaCl concentration which is typically between 0 and 1M NaCl, such as 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 or 1.0.

Chaotropic agents which disturb the secondary and tertiary structure of proteins, for example, guanidine salts such as guanidine hydrochloride (GnHCl) and thiocyanate (GnSCN), or urea, lithium chloride and other thiocyanates may be used in combination with detergents and reducing agents such as beta-mercaptoethanol or DTT to dissociate natural occurring nucleic acids and inhibit nucleases. The use of chaotropic agents in the extraction and hybridisation of nucleic acids is described in EP Publication No. 0 127 327, which is incorporated by reference herein.

An LNA substantially complementary to the target nucleic acid will be introduced in the hybridisation process. The term "an LNA substantially complementary to the target nucleic acid" refers to a polynucleotide or oligonucleotide containing at least one LNA monomer and a variable number of naturally occurring nucleotides or their analogues, such as 7-deazaguanosine or inosine, sufficiently complementary to hybridise with the target nucleic acid such that stable and specific binding occurs between the target and the complementary nucleic acid under the hybridisation conditions. Therefore, the LNA sequence need not reflect the exact sequence of the target nucleic acid. For example, a non-complementary nucleotide fragment may be attached to a complementary nucleotide fragment or alternatively, non-complementary bases or longer sequences can be interspersed into the complementary nucleic acid, provided that the complementary nucleic acid sequence has sufficient complementarity with the sequence of the target nucleic acid to hybridise therewith, forming a hybridisation complex and further is capable of immobilizing the target nucleic acid to a solid support as will be described in further detail below. A capturing probe to bind the released nucleic acids can be linked to a group (e.g. biotin, fluorescein, magnetic microparticle etc.). Alternatively, the capturing probe can be permanently bound to a solid phase or particle in advance e.g. by anthraquinone photochemistry (WO 96/31557).

An attractive possibility of the invention is the use of different LNA-oligomers directed against different sequences in the genome which are spotted in an array format and permanently affixed to the surface (Nature Genetics, suppl. vol. 21, January 1999, 1–60 and WO 96/31557). Such an array can subsequently be incubated with the mixture of the lysis buffer/hybridisation medium containing dissolved cells and a number of suitable detection LNA-probes. The lysis and hybridisation would then be allowed to occur, and finally the array would be washed and appropriately developed. The result of such a procedure would be a semi-quantitative assessment of a large number of different target nucleic acids.

As for DNA or RNA the degree of complementarity required for formation of a stable hybridisation complex (duplex) which includes LNA varies with the stringency of the hybridisation medium and/or wash medium. The complementary nucleic acid may be present in a pre-prepared hybridisation medium or introduced at some later point prior to hybridisation.

The hybridisation medium is combined with the biological sample to facilitate lysis of the cells and nucleic acid pairing. Preferably, the volume of biological sample to the volume of the hybridisation medium will be about 1:10.

It is intended and an advantage of the hybridisation methods of the present invention that they be carried out in one step on complex biological samples. However, minor mechanical or other treatments may be considered under certain circumstances. For example, it may be desirable to clarify the lysate before hybridisation such as by slow speed centrifugation or filtration or to extract the nucleic acids before hybridisation as described above.

The hybridisation assay of the present invention can be performed by any method known to those skilled in the art or analogous to immunoassay methodology given the guidelines presented herein. Preferred methods of assay are the sandwich assays and variations thereof and the competition or displacement assay. Hybridization techniques are generally described in "Nucleic Acid Hybridization, A Practical Approach," Ed. Hames, B. D. and Higgins, S. J., IRL Press, 1985; Gall and Pardue (1969), Proc. Natl. Acad. Sci., U.S.A., 63:378–383; and John, Burnsteil and Jones (1969) Nature, 223:582–587. Further improvements in hybridisation techniques will be well known to the person of skill in the art and can readily be applied.

In this invention the capturing LNA-probe is typically attached to a solid surface e.g. the surface of a microtiter tray well or a microbead. Therefore a convenient and very efficient washing procedure can be performed thus opening the possibility for various enzymatically based reactions that may add to the performance of the invention. Most noteworthy is the possibility that the sensitivity of the hybridisation assays may be enhanced through use of a nucleic acid amplification system which multiplies the target nucleic acid being detected. Examples of such systems include the polymerase chain reaction (PCR) system and the ligase chain reaction (LCR) system. Other methods recently described and known to the person of skill in the art are the nucleic acid sequence based amplification (NASBA™, Cangene, Mississauga, Ontario) and Q Beta Replicase systems. PCR is a template dependent DNA polymerase primer extension method of replicating selected sequences of DNA. The method relies upon the use of an excess of specific primers to initiate DNA polymerase replication of specific subsequences of a DNA polynucleotide followed by repeated denaturation and polymerase extension steps. The PCR system is well known in the art (see U.S. Pat. Nos. 4,683,195 and 4,683,202). For additional information regarding PCR methods, see also PCR Protocols: A Guide to Methods and Applications, ed. Innis, Gelland, Shinsky and White, Academic Press, Inc. (1990). Reagents and hardware for conducting PCR are available commercially through Perkin-Elmer/Cetus Instruments of Norwalk, Conn.

LCR, like PCR, uses multiple cycles of alternating temperature to amplify the numbers of a targeted sequence of DNA. LCR, however, does not use individual nucleotides for template extension. Instead, LCR relies upon an excess of oligonucleotides which are complementary to both strands of the target region. Following the denaturation of a double stranded template DNA, the LCR procedure begins with the ligation of two oligonucleotide primers complementary to adjacent regions on one of the target strands. Oligonucleotides complementary to either strand can be joined. After ligation and a second denaturation step, the original template strands and the two newly joined products serve as templates for additional ligation to provide an exponential amplification of the targeted sequences.

This method has been detailed in Genomics, 4:560–569 (1989), which is incorporated herein by reference. As other amplification systems are developed, they may also find use in this invention.

The hybridisation medium and processes of the present invention are uniquely suited to a one-step assay. The medium may be pre-prepared, either commercially or in the laboratory to contain all the necessary components for hybridisation. For instance, in a sandwich assay the medium could comprise a chaotropic agent (e.g. guanidine thiocyanate), desired buffers and detergents, a capturing LNA-probe bound to a solid support such as a microbead, and a detecting nucleic acid which could also be an LNA. This medium then only needs to be combined with the sample containing the target nucleic acid at the time the assay is to be performed. Once hybridisation occurs the hybridisation complex attached to the solid support may be washed and the extent of hybridisation determined.

Sandwich assays are commercially useful hybridisation assays for detecting or isolating nucleic acid sequences.

Such assays utilize a "capturing" nucleic acid covalently immobilized to a solid support and labelled "signal" nucleic acid in solution. The sample will provide the target nucleic acid. The "capturing" nucleic acid and "signal" nucleic acid probe hybridise with the target nucleic acid to form a "sandwich" hybridisation complex. To be effective, the signal nucleic acid is designed so that it cannot hybridise with the capturing nucleic acid, but will hybridise with the target nucleic acid in a different position than the capturing probe.

Virtually any solid surface can be used as a support for hybridisation assays, including metals and plastics. Two types of solid surfaces are generally available, namely:

a) Membranes, polystyrene beads, nylon, Teflon, polystyrene/latex beads, latex beads or any solid support possessing an activated carboxylate, sulfonate, phosphate or similar activatable group are suitable for use as solid surface substratum to which nucleic acids or oligonucleotides can be immobilized.

b) Porous membranes possessing pre-activated surfaces which may be obtained commercially (e.g., Pall Immunodyne Immunoaffinity Membrane, Pall BioSupport Division, East Hills, N.Y., or Immobilon Affinity membranes from Millipore, Bedford, Mass.) and which may be used to immobilize capturing oligonucleotides. Microbeads, including magnetic beads, of polystyrene, teflon, nylon, silica or latex may also be used.

However, use of the generally available surfaces mentioned in a) and b) often creates background problems, especially when complex mixtures of nucleic acids and various other dissolved bio-molecules are analysed by hybridisation. A significant decrease in the background has been obtained when the catching-probe is covalently attached to solid surfaces by the anthraquinone (AQ) based photo-coupling method described in the art (see WO 96/31557). This method allows the covalent attachment of the catching LNA-oligo to the surface of most polymer materials—including various relatively thermostable polymers such as polycarbonate and polyethylene—as well as treated glass surfaces. Thus by use of the AQ photo-coupling method, the capturing LNA-probe can be attached to surfaces of containers that is compatible with present days PCR amplification techniques.

Sequences suitable for capturing or signal nucleic acids for use in hybridisation assays can be obtained from the entire sequence or portions thereof of an organism's genome, from messenger RNA, or from cDNA obtained by reverse transcription of messenger RNA. Methods for obtaining the nucleotide sequence from such obtained sequences are well known in the art (see Ausubel et. al in Current Protocols in Molecular Biology, pub. John Wiley & Sons (1998), and Sambrook et al. in Molecular Cloning, A Laboratory Manual, Cold Spring Habor Laboratory Press, 1989). Furthermore, a number of both public and commercial sequence databases are accessible and can be approached to obtain the relevant sequences.

Once the appropriate sequences are determined, LNA probes are preferably chemically synthesized using commercially available methods and equipment as described in the art (Tetrahedron, 1998, 54, 3607–30.). For example, the solid phase phosphoramidite method can be used to produce short LNA probes. (Caruthers et al., Cold Spring Harbor Symp. Quant. Biol., 47:411–418 (1982), and Adams et al., J. Am. Chem. Soc., 105:661 (1983).

When synthesizing a probe for a specific target, the choice of nucleotide sequence will determine the specificity of the test. For example, by comparing DNA sequences from several virus isolates, one can select a sequence for virus detection that is either type specific or genus specific. Comparisons of DNA regions and sequences can be achieved using commercially available computer programmes.

The determination of the extent of hybridisation may be carried out by any of the methods well-known in the art. If there is no detectable hybridisation, the extent of hybridisation is thus 0. Typically, labelled signal nucleic acids are used to detect hybridisation. Complementary nucleic acids or signal nucleic acids may be labelled by any one of several methods typically used to detect the presence of hybridised polynucleotides. The most common method of detection is the use of ligands which bind to labelled antibodies, fluorophores or chemiluminescent agents. However, probes may also be labelled with ^{3}H, ^{125}I, ^{35}S ^{14}C, ^{33}P or ^{32}P and subsequently detected by autoradiography. The choice of radioactive isotope depends on research preferences due to ease of synthesis, varying stability, and half lives of the selected isotopes. Other labels include antibodies which can serve as specific binding pair members for a labelled ligand. The choice of label depends on sensitivity required, ease of conjugation with the probe, stability requirements, and available instrumentation.

LNA-probes are typically labelled during synthesis. The flexibility of the phosphoramidite synthesis approach furthermore facilitates the easy production of LNAs carrying all commercially available linkers, fluorophores and labelling-molecules available for this standard chemistry. LNA may also be labelled by enzymatic reactions e.g. by kinasing.

Situations can be envisioned in which the detection probes are DNA or RNA. Such probes can be labelled in various ways depending on the choice of label. Radioactive probes are typically made by using commercially available nucleotides containing the desired radioactive isotope. The radioactive nucleotides can be incorporated into probes by several means such as by nick translation of double-stranded probes; by copying single-stranded M13 plasmids having specific inserts with the Klenow fragment of DNA polymerase in the presence of radioactive dNTP; by transcribing cDNA from RNA templates using reverse transcriptase in the presence of radioactive dNTP; by transcribing RNA from vectors containing SP6 promoters or T7 promoters using SP6 or T7 RNA polymerase in the presence of radioactive rNTP; by tailing the 3' ends of probes with radioactive nucleotides using terminal transferase; or by phosphorylation of the 5' ends of probes using [^{32}P]-ATP and polynucleotide kinase.

Non-radioactive probes are often labelled by indirect means. Generally, a ligand molecule is covalently bound to the probe. The ligand then binds to an anti-ligand molecule which is either inherently detectable or covalently bound to a signal system, such as a detectable enzyme, a fluorescent compound, or a chemiluminescent compound. Ligands and antiligands may be varied widely. Where a ligand has a natural anti-ligand, for example, biotin, thyroxine, and cortisol, it can be used in conjunction with the labelled, naturally occurring anti-ligands. Alternatively, any haptenic or antigenic compound can be used in combination with an antibody.

As is the case of DNA, LNA-probes can also be conjugated directly to signal generating compounds, e.g., by conjugation with an enzyme or fluorophore. Enzymes of interest as labels will primarily be hydrolases, particularly phosphatases, esterases and glycosidases, or oxidoreductases, particularly peroxidases. Fluorescent com-

pounds include fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, etc. Chemiluminescent compounds include luciferin, AMPPD ([3-(2'-spiroamantane)4-methoxy4-(3'-phosphoryloxy)-phenyl-1,2-dioxetane]) and 2,3-dihydrophthalazinediones, e.g., luminol.

The amount of labelled probe which is present in the hybridisation medium or extraction solution may vary widely. Generally, substantial excesses of probe over the stoichiometric amount of the target nucleic acid will be employed to enhance the rate of binding of the probe to the target DNA. Treatment with ultrasound by immersion of the reaction vessel into commercially available sonication baths can often accelerate the hybridisation rates.

After hybridisation at a temperature and time period appropriate for the particular hybridisation solution used, the support to which the capturing LNA-probe:target nucleic acid hybridisation complex is attached is introduced into a wash solution typically containing similar reagents (e.g., sodium chloride, buffers, organic solvents and detergent), as provided in the hybridisation solution. These reagents may be at similar concentrations as the hybridisation medium, but often they are at lower concentrations when more stringent washing conditions are desired. The time period for which the support is maintained in the wash solutions may vary from minutes to several hours or more.

Either the hybridisation or the wash medium can be stringent. After appropriate stringent washing, the correct hybridisation complex may now be detected in accordance with the nature of the label.

The probe may be conjugated directly with the label. For example, where the label is raioactive, the probe with associated hybridisation complex substrate is exposed to X-ray film. Where the label is fluorescent, the sample is detected by first irradiating it with light of a particular wavelength. The sample absorbs this light and then emits light of a different wavelength which is picked up by a detector (Physical Biochemistry, Freifelder, D., W. H. Freeman & Co. (1982), pp. 537–542). Where the label is an enzyme, the sample is detected by incubation on an appropriate substrate for the enzyme. The signal generated may be a coloured precipitate, a coloured or fluorescent soluble material, or photons generated by bioluminescence or chemiluminescence. The preferred label for probe assays generates a coloured precipitate to indicate a positive reading, e.g. horseradish peroxidase, alkaline phosphatase, calf intestine alkaline phosphatase, glucose oxidase and beta-galactosidase. For example, alkaline phosphatase will dephosphorylate indoxyl phosphate which will then participate in a reduction reaction to convert tetrazolium salts to highly coloured and insoluble formazans.

Detection of a hybridisation complex may require the binding of a signal generating complex to a duplex of target and probe polynucleotides or nucleic acids. Typically, such binding occurs through ligand and anti-ligand interactions as between a ligand-conjugated probe and an anti-ligand conjugated with a signal. The binding of the signal generation complex is also readily amenable to accelerations by exposure to ultrasonic energy.

The label may also allow indirect detection of the hybridisation complex. For example, where the label is a hapten or antigen, the sample can be detected by using antibodies. In these systems, a signal is generated by attaching fluorescent or enzyme molecules to the antibodies or in some cases, by attachment to a radioactive label. (Tijssen, P., "Practice and Theory of Enzyme Immunoassays," Laboratory Techniques in Biochemistry and Molecular Biology, Burdon, R. H., van Knippenberg, P. H., Eds., Elsevier (1985), pp. 9–20.)

In the present context, the term "label" thus means a group which is detectable either by itself or as a part of an detection series. Examples of functional parts of reporter groups are biotin, digoxigenin, fluorescent groups (groups which are able to absorb electromagnetic radiation, e.g. light or X-rays, of a certain wavelength, and which subsequently reemits the energy absorbed as radiation of longer wavelength; illustrative examples are dansyl (5-dimethylamino)-1-naphthalenesulfonyl), DOXYL (N-oxyl4,4-dimethyloxazolidine), PROXYL (N-oxyl-2,2,5,5-tetramethylpyrrolidine), TEMPO (N-oxyl-2,2,6,6-tetramethylpiperidine), dinitrophenyl, acridines, coumarins, Cy3 and Cy5 (trademarks for Biological Detection Systems, Inc.), erytrosine, coumaric acid, umbelliferone, Texas Red, rhodamine, tetramethyl rhodamine, Rox, 7-nitrobenzo-2-oxa-1-diazole (NBD), pyrene, fluorescein, Europium, Ruthenium, Samarium, and other rare earth metals), radioisotopic labels, chemiluminescence labels (labels that are detectable via the emission of light during a chemical reaction), spin labels (a free radical (e.g. substituted organic nitroxides) or other paramagnetic probes (e.g. Cu^{2+}, Mg^{2+}) bound to a biological molecule being detectable by the use of electron spin resonance spectroscopy), enzymes (such as peroxidases, alkaline phosphatases, β-galactosidases, and glycose oxidases), antigens, antibodies, haptens (groups which are able to combine with an antibody, but which cannot initiate an immune response by themselves, such as peptides and steroid hormones), carrier systems for cell membrane penetration such as: fatty acid residues, steroid moieties (cholesteryl), vitamin A, vitamin D, vitamin E, folic acid peptides for specific receptors, groups for mediating endocytose, epidermal growth factor (EGF), bradykinin, and platelet derived growth factor (PDGF). Especially interesting examples are biotin, fluorescein, Texas Red, rhodamine, dinitrophenyl, digoxigenin, Ruthenium, Europium, Cy5, Cy3, etc.

In regard to the isolation of RNA, it has been described (U.S. Pat. No. 5,376,529) that a chaotropic agent, such as a salt of isothiocyanate (e.g. guanidine thiocyanate) does not provide for the complete disruption of protein and nucleic acid interactions, and thus prevents optimal hybridisation. A significant increase in hybridisation was reported to occur when heat is applied to the hybridisation solution containing the chaotropic agent and target nucleic acid. Previously, researchers have attempted to keep hybridisation temperatures low to maintain stability of the reactants. See Cox et al., EP Application No. 84302865.5. However, the significantly increased thermal stability of LNA/DNA and LNA/RNA heteroduplexes makes hybridisation with LNA-probes feasible at elevated temperatures. Thus the present invention provides a method for increasing the sensitivity of ribonucleic acid detection assays and for simplifying the steps of the assays. The processes for conducting nucleic acid hybridisations wherein the target nucleic acid is RNA comprise heating a nucleic acid solution or sample to an elevated temperature e.g. 70–100° C. as described in the art (U.S. Pat. No. 5,376,529). The nucleic acid solution of the present invention will comprise a chaotropic agent, a target nucleic acid, and an LNA substantially complementary to the target nucleic acid of interest. The nucleic acid solution will be heated to fully disrupt the protein and nucleic acid interactions to maximize hybridisation between the LNA and its target.

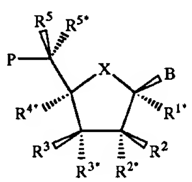
When very high affinity LNA probes are used, hybridisation may take place even at the increased temperature needed to fully disrupt DNA:DNA and DNA:RNA interactions. The solution is then cooled until the complementary

nucleic acid has hybridised with the target nucleic acid to form a hybridisation complex.

These methods are additionally advantageous because they allow for minimal handling of the samples and assay reagents. A ready-to-use reagent solution may be provided, for example, which would contain a chaotropic agent, other appropriate components such as buffers or detergents, a capturing LNA-probe bound to a solid support, and a signal or detection LNA (or nucleic acid), both capable of hybridising with a target nucleic acid. Conveniently, a complex biological sample suspected of harbouring a target nucleic acid can be directly combined with the pre-prepared reagent for hybridisation, thus allowing the hybridisation to occur in one step. The combined solution is heated as described herein and then cooled until hybridisation has occurred. The resulting hybridisation complex is then simply washed to remove unhybridised material, and the extent of hybridisation is determined.

Kits for the extraction of and hybridisation of nucleic acids, e.g. mRNA, are also contemplated. Such kits would contain at least one vial containing an extraction solution or a hybridisation medium which comprises a strong chaotropic agent and a capturing LNA-probe bound to a solid support. Detergents, buffer solutions and additional vials which contain components to detect target nucleic acids may also be included.

When used herein, the terms "LNA" or "capturing LNA-probe" refer to oligomers comprising at least one nucleoside analogue of the general formula I



I

wherein

- X is selected from —O—, —S—, —N(R^{N*})—, —CR^6(R^{6*})—;
- B is selected from nucleobases;
- P designates the radical position for an internucleoside linkage to a succeeding monomer, or a 5'-terminal group, such internucleoside linkage or 5'-terminal group optionally including the substituent R^5;
- R^3 or R^{3*} is P* which designates an internucleoside linkage to a preceding monomer, or a 3'-terminal group;
- R^{4*} and R^{2*} together designate a biradical consisting of 1-4 groups/atoms selected from —C(R^aR^b)—, —C(R^a)=C(R^a)—, —C(R^a)=N—, —O—, —Si(R^a)$_2$—, —S—, —SO_2—, —N(R^a)—, and>C=Z,

wherein Z is selected from —O—, —S—, and —N(R^a)—, and R^a and R^b each is independently selected from hydrogen, optionally substituted C_{1-12}-alkyl, optionally substituted C_{2-12}-alkenyl, optionally substituted C_{2-12}-alkynyl, hydroxy, C_{1-12}-alkoxy, C_{2-12}-alkenyloxy, carboxy, C_{1-12}-alkoxycarbonyl, C_{1-12}-alkylcarbonyl, formyl, aryl, aryloxycarbonyl, aryloxy, arylcarbonyl, heteroaryl, heteroaryloxy-carbonyl, heteroaryloxy, heteroarylcarbonyl, amino, mono- and di(C_{1-6}alkyl)amino, carbamoyl, mono- and di(C_{1-6}-alkyl)-amino-carbonyl, amino-C_{1-6}-alkyl)-aminocarbonyl, mono- and di(C_{1-6}-alkyl)amino-C_{1-6}-alkyl) amino-C_{1-6}-alkyl-aminocarbonyl, C_{1-6}-alkyl-carbonylamino, carbamido, C_{1-6}-alkanoyloxy, sulphono, C_{1-6}-alkylsulphonyloxy, nitro, azido, sulphanyl, C_{1-6}-alkylthio, halogen, DNA intercalators, photochemically active groups, thermochemically active groups, chelating groups, reporter groups, and ligands, where aryl and heteroaryl may be optionally substituted, and where two geminal substituents R^a and R^b together may designate optionally substituted methylene (=CH_2, optionally substituted one or two times with substituents as defined as optional substituents for aryl); and

- each of the substituents R^{1*}, R^2, R^3, R^{3*}, R^5, R^{5*}, R^6 and R^{6*} which are present and not involved in P or P*, is independently selected from hydrogen, optionally substituted C_{1-12}-alkyl, optionally substituted C_{2-12}-alkenyl, optionally substituted C_{2-12}-alkynyl, hydroxy, C_{1-12}-alkoxy, C_{2-12}-alkenyloxy, carboxy, C_{1-12}-alkoxycarbonyl, C_{1-12}-alkylcarbonyl, formyl, aryl, aryloxy-carbonyl, aryloxy, arylcarbonyl, heteroaryl, heteroaryloxy-carbonyl, heteroaryloxy, heteroarylcarbonyl, amino, mono- and di(C_{1-6}-alkyl) amino, carbamoyl, mono- and di(C_{1-6}alkyl)-amino-carbonyl, amino-C_{1-6}-alkyl-aminocarbonyl, mono- and di(C_{1-6}-alkyl)amino-C_{1-6}-alkyl-aminocarbonyl, C_{1-6}-alkyl-carbonylamino, carbamido, C_{1-6}-alkanoyloxy, sulphono, C_{1-6}-alkylsulphonyloxy, nitro, azido, sulphanyl, C_{1-6}-alkylthio, halogen, DNA intercalators photochemically active groups, thermochemically active groups, chelating groups, reporter groups, and ligands (where the latter groups may include a spacer as defined for the substituent B), where aryl and heteroaryl may be optionally substituted, and where two geminal substituents together may designate oxo, thioxo, imino, or optionally substituted methylene, or together may form a spiro biradical consisting of a 1–5 carbon atom(s) alkylene chain which is optionally interrupted and/or terminated by one or more heteroatoms/groups selected from —O—, —S—, and —(NR^N) — where R^N is selected from hydrogen and C_{1-4}-alkyl, and where two adjacent (non-geminal) substituents may designate an additional bond resulting in a double bond; and R^{N*}, when present and not involved in a biradical, is selected from hydrogen and C_{1-4}-alkyl;

and basic salts and acid addition salts thereof.

When used herein, the term "LNA" (Locked Nucleoside Analogues) refers to the bi-cyclic nucleoside analogues incorporated in the oligomer (general formula I).

In the present context, the term "nucleobase" covers naturally occurring nucleobases as well as non-naturally occurring nucleobases. It should be clear to the person skilled in the art that various nucleobases which previously have been considered "non-naturally occurring" have subsequently been found in nature. Thus, "nucleobase" includes not only the known purine and pyrimidine heterocycles, but also heterocyclic analogues and tautomers thereof. Illustrative examples of nucleobases are adenine, guanine, thymine, cytosine, uracil, purine, xanthine, diaminopurine, 8-oxo-N^6-methyladenine, 7-deazaxanthine, 7-deazaguanine, N^4,N^4-ethanocytosine, N^6, N^6-ethano-2,6-diaminopurine, 5-methylcytosine, 5-(C^3—C^6)-alkynylcytosine, 5-fluorouracil, 5-bromouracil, pseudoisocytosine, 2-hydroxy-5-methyl4-triazolopyridine, isocytosine, isoguanine, inosine and the "non-naturally occurring" nucleobases described in Benner et al., U.S. Pat. No. 5,432,272. The term "nucleobase" is intended to cover every and all of these examples as well as analogues and tautomers thereof. Especially interesting nucleobases are adenine, guanine, thymine, cytosine, and uracil, which are considered as the naturally occurring nucleobases in relation to therapeutic and diagnostic applications in humans.

When used herein, the term "DNA intercalator" means a group which can intercalate into a DNA or RNA helix, duplex or triplex. Examples of functional parts of DNA intercalators are acridines, anthracene, quinones such as anthraquinone, indole, quinoline, isoquincline, dihydroquinones, anthracyclines, tetracyclines, methylene blue, anthracyclinone, psoralens, coumarins, ethidium-halides, dynemicin, metal complexes such as 1,10-phenanthroline-copper, tris(4,7-diphenyl-1,10-phenanthroline)ruthenium-cobalt-enediynes such as calcheamicin, porphyrins, distamycin, netropcin, viologen, daunomycin. Especially interesting examples are acridines, quinones such as anthraquinone, methylene blue, psoralens, coumarins, and ethidium-halides.

In the present context, the term "photochemically active groups" covers compounds which are able to undergo chemical reactions upon irradiation with light. Illustrative examples of functional groups hereof are quinones, especially 6-methyl-1,4-naphthoquinone, anthraquinone, naphthoquinone, and 1,4-dimethyl-anthraquinone, diazirines, aromatic azides, benzophenones, psoralens, diazo compounds, and diazirino compounds.

In the present context "thermochemically reactive group" is defined as a functional group which is able to undergo thermochemically induced covalent bond formation with other groups. Illustrative examples of functional parts of thermochemically reactive groups are carboxylic acids, carboxylic acid esters such as activated esters, carboxylic acid halides such as acid fluorides, acid chlorides, acid bromide, and acid iodides, carboxylic acid azides, carboxylic acid hydrazides, sulfonic acids, sulfonic acid esters, sulfonic acid halides, semicarbazides, thiosemicarbazides, aldehydes, ketones, primary alcohols, secondary alcohols, tertiary alcohols, phenols, alkyl halides, thiols, disulphides, primary amines, secondary amines, tertiary amines, hydrazines, epoxides, maleimides, and boronic acid derivatives.

In the present context, the term "chelating group" means a molecule that contains more than one binding site and frequently binds to another molecule, atom or ion through more than one binding site at the same time. Examples of functional parts of chelating groups are iminodiacetic acid, nitrilotriacetic acid, ethylenediamine tetraacetic acid (EDTA), aminophosphonic acid, etc.

In the present context "ligand" means something which binds. Ligands can comprise functional groups such as: aromatic groups (such as benzene, pyridine, naphthalene, anthracene, and phenanthrene), heteroaromatic groups (such as thiophene, furan, tetrahydrofuran, pyridine, dioxane, and pyrimidine), carboxylic acids, carboxylic acid esters, carboxylic acid halides, carboxylic acid azides, carboxylic acid hydrazides, sulfonic acids, sulfonic acid esters, sulfonic acid halides, semicarbazides, thiosemicarbazides, aldehydes, ketones, primary alcohols, secondary alcohols, tertiary alcohols, phenols, alkyl halides, thiols, disulphides, primary amines, secondary amines, tertiary amines, hydrazines, epoxides, maleimides, C_1—C_{20} alkyl groups optionally interrupted or terminated with one or more heteroatoms such as oxygen atoms, nitrogen atoms, and/or sulphur atoms, optionally containing aromatic or mono/polyunsaturated hydrocarbons, polyoxyethylene such as polyethylene glycol, oligo/polyamides such as poly-β-alanine, polyglycine, polylysine, peptides, oligo/polysaccharides, oligo/polyphosphates, toxins, antibiotics, cell poisons, and steroids, and also "affinity ligands", i.e. functional groups or biomolecules that have a specific affinity for sites on particular proteins, antibodies, poly- and oligosaccharides, and other biomolecules.

It will be clear for the person skilled in the art that the above-mentioned specific examples of DNA intercalators, photochemically active groups, thermochemically active groups, chelating groups, reporter groups, and ligands correspond to the "active/functional" part of the groups in question. For the person skilled in the art it is furthermore clear that DNA intercalators, photochemically active groups, thermochemically active groups, chelating groups, reporter groups, and ligands are typically represented in the form M—K—where M is the "active/functional" part of the group in question and where K is a spacer through which the "active/functional" part is attached to the 5- or 6-membered ring. Thus, it should be understood that the group B, in the case where B is selected from DNA intercalators, photochemically active groups, thermochemically active groups, chelating groups, reporter groups, and ligands, has the form M—K—, where M is the "active/functional" part of the DNA intercalator, photochemically active group, thermochemically active group, chelating group, reporter group, and ligand, respectively, and where K is an optional spacer comprising 1–50 atoms, preferably 1–30 atoms, in particular 1–15 atoms, between the 5- or 6-membered ring and the "active/functional" part.

In the present context, the term "spacer" means a thermochemically and photochemically non-active distance-making group which is used to join two or more different moieties of the types defined above. Spacers are selected on the basis of a variety of characteristics including their hydrophobicity, hydrophilicity, molecular flexibility and length (e.g. see Hermanson et. al., "Immobilized Affinity Ligand Techniques", Academic Press, San Diego, Calif. (1992), p. 137-ff). Generally, the length of the spacers is less than or about 400 Å, in some applications preferably less than 100 A. The spacer, thus, comprises a chain of carbon atoms optionally interrupted or terminated with one or more heteroatoms, such as oxygen atoms, nitrogen atoms, and/or sulphur atoms. Thus, the spacer K may comprise one or more amide, ester, amino, ether, and/or thioether functionalities, and optionally aromatic or mono/polyunsaturated hydrocarbons, polyoxyethylene such as polyethylene glycol, oligo/polyamides such as poly-β-alanine, polyglycine, polylysine, and peptides in general, oligosaccharides, oligo/polyphosphates. Moreover the spacer may consist of combined units thereof. The length of the spacer may vary, taking into consideration the desired or necessary positioning and spatial orientation of the "active/functional" part of the group in question in relation to the 5- or 6-membered ring. In particularly interesting embodiments, the spacer includes a chemically cleavable group. Examples of such chemically cleavable groups include disulphide groups cleavable under reductive conditions, peptide fragments cleavable by peptidases, etc.

In one variant, K designates a single bond so that the "active/functional" part of the group in question is attached directly to the 5- or 6-membered ring.

In a preferred embodiment, the substituent B in the general formulae I and II is preferably selected from nucleobases, in particular from adenine, guanine, thymine, cytosine and uracil.

In the oligomers (formula I), P designates the radical position for an internucleoside linkage to a succeeding monomer, or a 5'-terminal group. The first possibility applies when the LNA in question is not the 5'-terminal "monomer", whereas the latter possibility applies when the LNA in question is the 5'-terminal "monomer". It should be understood (which will also be clear from the definition of internucleoside linkage and 5'-terminal group further below)

that such an internucleoside linkage or 5'-terminal group may include the substituent R^5 (or equally applicable: the substituent R^{5*}) thereby forming a double bond to the group P. (5'-Terminal refers to the position corresponding to the 5' carbon atom of a ribose moiety in a nucleoside.)

On the other hand, an internucleoside linkage to a preceding monomer or a 3'-terminal group (P*) may originate from the positions defined by one of the substituents R^3 or R^{3*}, preferably from the positions defined by the substituents R^{3*}. (3'-Terminal refers to the position corresponding to the 3' carbon atom of a ribose moiety in a nucleoside.)

It should be understood that the orientation of the group P* either as R^{3*} ("normal" configuration) or as R^3 (xylo configuration) represents two equally interesting possibilities. It has been found that all-"normal" (R^{3*}=P*) oligomers and oligomers with combinations of "normal" LNA monomers and nucleotides (2-deoxynucleotides and/or nucleotides) hybridise strongly (with increasing affinity) to DNA, RNA and other LNA oligomers. It is presently believed that combination of all-xylo LNA oligomers and oligomers with xylo LNA (R^3=P*) monomers and, e.g., xylo nucleotides (nucleotides and/or 2-deoxynucleotides) will give rise to comparable hybridisation properties. It has been shown that an oligomer with "normal" configuration (R^{3*}=P*) will give rise to an anti-parallel orientation of an LNA oligomer when hybridised (with increasing affinity) to either DNA, RNA or another LNA oligomer. It is thus contemplated that an oligomer with xylo configuration (R^3=P*) will give rise to a parallel orientation when hybridised to DNA, RNA or another LNA.

In view of the above, it is contemplated that the combination of "normal" LNAs and xylo-LNAs in one oligomer can give rise to interesting properties as long as these monomers of different type are located in domains, i.e. so that an uninterrupted domain of at least 5, such as at least 10, monomers (e.g. xylo-LNA, xylo-nucleotides, etc. monomers) is followed by an uninterrupted domain of at least 5, e.g. at least 10, monomers of the other type (e.g. "normal" LNA, "normal" nucleotides, etc.), etc. Such chimeric type oligomers may, e.g., be used to capture nucleic acids.

In the present context, the term "monomer" relates to naturally occurring nucleosides, non-naturally occurring nucleosides, PNAs, etc. as well as LNAs. Thus, the term "succeeding monomer" relates to the neighbouring monomer in the 5'-terminal direction and the "preceding monomer" relates to the neighbouring monomer in the 3'-terminal direction. Such succeeding and preceding monomers, seen from the position of an LNA monomer, may be naturally occurring nucleosides or non-naturally occurring nucleosides, or even further LNA monomers.

Consequently, in the present context (as can be derived from the definitions above), the term "oligomer" means an oligonucleotide modified by the incorporation of one or more LNA(s).

In the present context, the orientation of the biradical (R^{2*}—R^{4*}) is so that the left-hand side represents the substituent with the lowest number and the right-hand side represents the substituent with the highest number, thus, when R^{2*} and R^{4*} together designate a biradical "—O—CH_2—", it is understood that the oxygen atom represents R^{2*}, thus the oxygen atom is e.g. attached to the position of R^{2*}, and the methylene group represents R^{4*}.

Considering the numerous interesting possibilities for the structure of the biradical (R^{2*}—R^{4*}) in LNA(s) incorporated in oligomers, it is believed that the biradical is preferably selected from —(CR*R*)$_r$—Y—(CR*R*)$_s$—, —(CR*R*)$_r$—Y—(CR*R*)$_s$—Y—, —Y—(CR*R*)$_{r+s}$—Y—, —Y—(CR*R*)$_r$—Y—(CR*R*)$_s$—, —(CR*R*)$_{r+s}$—, —Y—, —Y—Y—, wherein each Y is independently selected from —O—, —S—, —Si(R*)$_2$—, —N(R*)—, >C=O, —C(=O)—N(R*)—, and —N(R*)—C(=O)—, wherein each R* is independently selected from hydrogen, halogen, azido, cyano, nitro, hydroxy, mercapto, amino, mono- or di(C_{1-6}-alkyl)amino, optionally substituted C_{1-6}-alkoxy, optionally substituted C_{1-6}-alkyl, DNA intercalators, photochemically active groups, thermochemically active groups, chelating groups, reporter groups, and ligands, and/or two adjacent (non-geminal) R* may together designate a double bond; and each of r and s is 0–4 with the proviso that the sum r+s is 1–4. Particularly interesting situations are those wherein the biradical is selected from —Y—, —(CR*R*)$_{r+s}$—, —(CR*R*)$_r$—Y—(CR*R*)$_s$—, and —Y—(CR*R*)$_{r+s}$—Y—, wherein and each of r and s is 0–3 with the proviso that the sum r+s is 1–4.

Particularly interesting oligomers are those wherein R^{2*} and R^{4*} in at least one LNA in the oligomer together designate a biradical selected from —O—, —S—, —N(R*)—, —(CR*R*)$_{r+s+1}$—, —(CR*R*)$_r$—O—(CR*R*)$_s$—, —(CR*R*)$_r$—S—(CR*R*)$_s$—, —(CR*R*)$_r$—N(R*)—(CR*R*)$_s$—, —O—(CR*R*)$_{r+s}$—O—, —S—(CR*R*)$_{r+s}$—O—, —O—(CR*R*)$_{r+s}$—S—, —N(R*)—(CR*R*)$_{r+s}$—O—, —O—(CR*R*)$_{r+s}$—N(R*)—, —S—(CR*R*)$_{r+s}$—S—, —N(R*)—(CR*R*)$_{r+s}$—N(R*)—, —N(R*)—(CR*R*)$_{r+s}$—S—, and —S —(CR*R*)$_{r+s}$—N(R*)—.

It is furthermore preferred that one R* is selected from hydrogen, hydroxy, optionally substituted C_{1-6}-alkoxy, optionally substituted C_{1-6}-alkyl, DNA intercalators, photochemically active groups, thermochemically active groups, chelating groups, reporter groups, and ligands, and any remaining substituents R* are hydrogen.

In one preferred variant, one group R* in the biradical of at least one LNA is selected from DNA intercalators, photochemically active groups, thermochemically active groups, chelating groups, reporter groups, and ligands (where the latter groups may include a spacer as defined for the substituent B).

Preferably, each of the substituents R^{1*}, R^2, R^3, R^{3*}, R^5, R^{5*}, R^6 and R^{6*} of the LNA(s), which are present and not involved in P or P*, is independently selected from hydrogen, optionally substituted C_{1-6}-alkyl, optionally substituted C_{2-6}-alkenyl, hydroxy, C_{1-6}-alkoxy, C_{2-6}-alkenyloxy, carboxy, C_{1-6}-alkoxycarbonyl, C_{1-6}-alkylcarbonyl, formyl, amino, mono- and di(C_{1-6}-alkyl)amino, carbamoyl, mono- and di(C_{1-6}-alkyl)-amino-carbonyl, C_{1-6}-alkyl-carbonylamino, carbamido, azido, C_{1-6}-alkanoyloxy, sulphono, sulphanyl, C_{1-6}-alkylthio, DNA intercalators, photochemically active groups, thermochemically active groups, chelating groups, reporter groups, and ligands, and halogen, where two geminal substituents together may designate oxo, and where R^{N*}, when present and not involved in a biradical, is selected from hydrogen and C_{1-4}-alkyl.

In a preferred variant of the LNAs, X is selected from —O—, —S—, and —NR^{N*}—, in particular —O—, and each of the substituents R^{1*}, R^2, R^3, R^{3*}, R^5, R^{5*}, R^6 and R^{6*} of the LNA(s), which are present and not involved in P or P*, designates hydrogen.

In an even more preferred variant, X is O, R^2 is selected from hydrogen, hydroxy, and optionally substituted C_{1-6}alkoxy, one of R^3 and R^{3*} is P* and the other is hydrogen, and R^{1*}, R^5, and R^{5*} designate hydrogen, and, more specifically, the biradical (R^{2*}—R^{4*}) is selected from

—O—, —$(CH_2)_{0-1}$—O—$(CH_2)_{1-3}$—, —$(CH_2)_{0-1}$—S—$(CH_2)_{1-3}$—, —$(CH_2)_{0-1}$—N(R^N)—$(CH_2)_{1-3}$—, and —$(CH_2)_{2-4}$—, in particular from —O—CH_2—, —S—CH_2—, and —NR^H—CH_2—. Generally, with due regard to the results obtained so far, it is preferred that the biradical constituting R^{2*} and R^{4*} forms a two carbon atom bridge, i.e. the biradical forms a five membered ring with the furanose ring (X=O). Particularly interesting are also those oligomers where R^{2*} and R^{4*} of an incorporated LNA of formula I together designate a biradical selected from —O—CH_2—, —S—CH_2—, and —NR^H—CH_2—; X is O, B designates a nucleobase selected from adenine, guanine, thymine, cytosine and uracil; R^2 is hydrogen, one of R^3 or R^{3*} designates P* and the other is hydrogen, and R^{1*}, R^3, R^5, and R^{5*} designate hydrogen.

In these embodiments, it is furthermore preferred that at least one LNA incorporated in an oligomer includes a nucleobase (substituent B) selected from adenine and guanine. In particular, it is preferred that an oligomer having LNA incorporated therein includes at least one nucleobase selected from thymine, uracil and cytosine and at least one nucleobase selected from adenine and guanine. For LNA monomers, it is especially preferred that the nucleobase is selected from adenine and guanine.

Within a variant of these interesting embodiments, all monomers of a oligonucleotide are LNA monomers.

As it will be evident from the general formula I (LNA(s) in an oligomer) and the definitions associated therewith, there may be one or several asymmetric carbon atoms present in the oligomers depending on the nature of the substituents and possible biradicals, cf. below.

In one variant, R^{3*} designates P*. In another variant, R^3 designates P*, and in a third variant, some R^{3*} designates P* in some LNAs and R^3 designates P* in other LNAs within an oligomer.

The oligomers typically comprise 1–10000 LNA(s) of the general formula I and 0–10000 nucleosides selected from naturally occurring nucleosides and nucleoside analogues. The sum of the number of nucleosides and the number of LNA(s) is at least 2, preferably at least 3, in particular at least 5, especially at least 7, such as in the range of 2–15000, preferably in the range of 2–100, such as 3–100, in particular in the range of 2–50, such as 3–50 or 5–50 or 7–50.

In the present context, the term "nucleoside" means a glycoside of a heterocyclic base. The term "nucleoside" is used broadly as to include non-naturally occurring nucleosides, naturally occurring nucleosides as well as other nucleoside analogues. Illustrative examples of nucleosides are ribonucleosides comprising a ribose moiety as well as deoxyribonuclesides comprising a deoxyribose moiety. With respect to the bases of such nucleosides, it should be understood that this may be any of the naturally occurring bases, e.g. adenine, guanine, cytosine, thymine, and uracil, as well as any modified variants thereof or any possible unnatural bases.

When considering the definitions and the known nucleosides (naturally occurring and nonnaturally occurring) and nucleoside analogues (including known bi- and tricyclic analogues), it is clear that an oligomer may comprise one or more LNA(s) (which may be identical or different both with respect to the selection of substituent and with respect to selection of biradical) and one or more nucleosides and/or nucleoside analogues. In the present context "oligonucleotide" means a successive chain of nucleosides connected via internucleoside linkages; however, it should be understood that a nucleobase in one or more nucleotide units (monomers) in an oligomer (oligonucleotide) may have been modified with a substituent B as defined above.

As mentioned above, the LNA(s) of an oligomer is/are connected with other monomers via an internucleoside linkage. In the present context, the term "internucleoside linkage" means a linkage consisting of 2 to 4, preferably 3, groups/atoms selected from —CH_2—, —O—, —S—, —NR^H—, >C=O, >C=NR^H, >C=S, —Si(R")$_2$—, —SO—, —S(O)$_2$—, —P(O)$_2$—, —PO(BH_3)—, —P(O,S)—, —P(S)$_2$—, —PO(R")—, —PO(OCH_3)—, and —PO(NHR^H)—, where R^H is selected form hydrogen and C_{1-4}-alkyl, and R" is selected from C_{1-6}-alkyl and phenyl. Illustrative examples of such internucleoside linkages are —CH_2—CH_2—CH_2—, —CH_2—CO—CH_2—, —CH_2—CHOH—CH_2—, —O—CH_2—O—, —O—CH_2—CH_2—, —O—CH_2—CH= (including R^5 when used as a linkage to a succeeding monomer), —CH_2—CH_2—O—, —NR^H—CH_2—CH_2—, —CH_2—CH_2—NR^H—, —CH_2—NR^H—CH_2—, —O—CH_2—CH_2—NR^H—, —NR^H—CO—O—, —NR^H—CO—NR^H—, —NR^H—CS—NR^H—, —NR^H—C(=NR^H) —NR^H—, —NR^H—CO—CH_2—NR^H—, —O—CO—O—, —O—CO—CH_2—O—, —O—CH_2—CO—O—, —CH_2—CO—NR^H—, —O—CO—NR^H—, —NR^H—CO—CH_2—, —O—CH_2—CO—NR^H—, —O—CH_2—NR^H—, —CH=N—O—, —CH_2—NR^H—O—, —CH_2—O—N= (including R^5 when used as a linkage to a succeeding monomer), —CH_2—O—NR^H—, —CO—NR^H—CH_2—, —CH_2—NR^H—O—, —CH_2—NR^H—CO—, —O—NR^H—CH_2—, —O—NR^H—, —O—CH_2—S—, —S—CH_2—O—, —CH_2—CH_2—S—, —O—CH_2—CH_2—S—, —S—CH_2—CH= (including R^5 when used as a linkage to a succeeding monomer), —S—CH_2—CH_2—, —S—CH_2—CH_2—O—, —S—CH_2—CH_2—S—, —CH_2—S—CH_2—, —CH_2—SO—CH_2—, —CH_2—SO_2—CH_2—, —O—SO—O—, —O—S(O)$_2$—O—, —O—S(O)$_2$—CH_2—, —O—S(O)$_2$—NR^H—, —NR^H—S(O)$_2$—CH_2—, —O—S(O)$_2$—CH_2—, —O—P(O)$_2$—O—, —O—P(O,S)—O—, —O—P(S)$_2$—O—, —S—P(O)$_2$—O—, —S—P(O,S)—O—, —S—P(S)$_2$—O—, —O—P(O)$_2$—S—, —O—P(O,S)—S—, —O—P(S)$_2$—S—, —S—P(O)$_2$—S —, —S—P(O,S)—S—, —S—P(S)$_2$—S—, —O—PO(R")—O—, —O—PO(OCH_3)—O—, —O—PO(OCH_2CH_3)—O—, —O—PO(OCH_2CH_2S —R)—O—, —O—PO(BH_3)—O—, —O—PO(NHR^N)—O—, —O—P(O)$_2$—NR^H, —NR^H—P(O)$_2$—O—, —O—P(O,NR^H)—O—, —CH_2—P(O)$_2$—O—, —O—P(O)$_2$—CH_2—, and —O—Si(R")$_2$—O—; among which —CH_2—CO—NR ^{H}—, —CH_2—NR^H—O—, —S—CH_2—O—, —O—P(O)$_2$—O—, —O—P(O,S)—O—, —O—P(S)$_2$—O—, —NR^H—P(O)$_2$—O—, —O—P (O,NR^H)—O—, —O—PO(R")—O—, —O—PO(CH_3)—O—, and —O—PO(NHR^N)—O—, where R^H is selected from hydrogen and C_{1-4}-alkyl, and R" is selected from C_{1-6}-alkyl and phenyl, are especially preferred. Further illustrative examples are given in Mesmaeker et. al., Current Opinion in Structural Biology 1995, 5, 343–355. The left-hand side of the internucleoside linkage is bound to the 5-membered ring as substituent P*, whereas the right-hand side is bound to the 5'-position of a preceding monomer.

It is also clear from the above that the group P may also designate a 5'-terminal group in the case where the LNA in question is the 5'-terminal monomer. Examples of such 5'-terminal groups are hydrogen, hydroxy, optionally substituted C_{1-6}-alkyl, optionally substituted C_{1-6}-alkoxy, optionally substituted C_{1-6}-alkylcarbonyloxy, optionally substituted aryloxy, monophosphate, diphosphate, triphosphate, and —W—A', wherein W is selected from —O—, —S—, and —N(R^H) where R^H is selected from hydrogen and C_{1-6}alkyl, and where A' is selected from DNA

intercalators, photochemically active groups, thermochemically active groups, chelating groups, reporter groups, and ligands (where the latter groups may include a spacer as defined for the substituent B).

In the present description and claims, the terms "monophosphate", "diphosphate", and "triphosphate" mean groups of the formula: —O—P(O)$_2$—O$^-$, —O—P(O)$_2$—O—P(O)$_2$—O$^-$, and —O—P(O)$_2$—O—P(O)$_2$—O—P(O)$_2$—O$^-$, respectively.

In a particularly interesting embodiment, the group P designates a 5'-terminal group selected from monophosphate, diphosphate and triphosphate. Especially the triphosphate variant is interesting as a substrate for nucleic acid polymerases.

Analogously, the group P* may designate a 3'-terminal group in the case where the LNA in question is the 3'-terminal monomer. Examples of such 3'-terminal groups are hydrogen, hydroxy, optionally substituted C_{1-6}-alkoxy, optionally substituted C_{1-6}-alkylcarbonyloxy, optionally substituted aryloxy, and —W—A', wherein W is selected from —O—, —S—, and —N(R^H)—where R^H is selected from hydrogen and C_{1-6}-alkyl, and where A' is selected from DNA intercalators, photochemically active groups, thermochemically active groups, chelating groups, reporter groups, and ligands (where the latter groups may include a spacer as defined for the substituent B).

In a preferred variant of the LNAs, the oligomer has the following formula V:

$$G—[NU—L]_{n(o)}—\{[LNA—L]_{m(q)}—[Nu—L]_{n(q)}\}_q—G^* \quad V$$

wherein

q is 1–50;

each of n(0), . . . , n(q) is independently 0–10000;

each of m(1), . . . , m(q) is independently 1–10000;

with the proviso that the sum of n(0), . . . , n(q) and m(1), . . . , m(q) is 2–15000;

G designates a 5'-terminal group;

each Nu independently designates a nucleoside selected from naturally occurring nucleosides and nucleoside analogues;

each LNA independently designates a nucleoside analogue;

each L independently designates an internucleoside linkage between two groups selected from Nu and LNA, or L together with G* designates a 3'-terminal group; and

each LNA-L independently designates a nucleoside analogue of the general formula I as defined above.

Within this variant, as well as generally, the LNAs preferably include different nucleobases, in particular both nucleobases selected from thymine, cytosine and uracil and nucleobases selected from adenine and guanine.

The oligomers are also intended to cover chimeric oligomers. The term "chimeric oligomers" means two or more oligomers with monomers of different origin joined either directly or via a spacer. Illustrative examples of such oligomers which can be combined are peptides, PNA-oligomers, oligomers containing LNAs, and oligonucleotide oligomers. The combination of an oligomer having xylo-LNA (R^3=P*) domain(s) and "normal" LNA (R^{3*}=P*) domain(s) might be construed as an example of a chimeric oligomer as the various domains may have different affinity and specificity profiles.

Generally, the oligomers have surprisingly good hybridisation properties with respect to affinity and specificity. Thus, the oligomers comprise at least one nucleoside analogue which imparts to the oligomer a T_m with a complementary DNA oligonucleotide which is at least 2.5° C. higher, preferably at least 3.5° C. higher, in particular at least 4.0° C. higher, especially at least 5.0° C. higher, than that of the corresponding unmodified reference oligonucleotide which does not comprise any nucleoside analogue. In particular, the T_m of the oligomer is at least 2.5×N° C. higher, preferably at least 3.5×N° C. higher, in particular at least 4.0×N° C. higher, especially at least 5.0×N ° C. higher, where N is the number of nucleoside analogues.

In the case of hybridisation with a complementary RNA oligonucleotide, the at least one nucleoside analogue imparts to the oligomer a T_m with the complementary DNA oligonucleotide which is at least 4.0° C. higher, preferably at least 5.0° C. higher, in particular at least 6.0° C. higher, especially at least 7.0° C. higher, than that of the corresponding un-modified reference oligonucleotide which does not comprise any nucleoside analogue. In particular, the T_m of the oligomer is at least 4.0×N° C. higher, preferably at least 5.0×N° C. higher, in particular at least 6.0×N° C. higher, especially at least 7.0×N° C. higher, where N is the number of nucleoside analogues.

The term "corresponding unmodified reference oligonucleotide" is intended to mean an oligonucleotide solely consisting of naturally occurring nucleotides which represents the same nucleobases in the same absolute order (and the same orientation).

The T_m is measured under one of the following conditions:

a) 10 mM Na_2HPO_4, pH 7.0, 100 mM NaCl, 0.1 mM EDTA;

b) 10 mM Na_2HPO_4 pH 7.0, 0.1 mM EDTA; or

c) 3 M tetramethylammoniumchloride (TMAC), 10 mM Na_2HPO_4, pH 7.0, 0.1 mM EDTA;

preferably under conditions a), at equimolar amounts (typically 1.0 μM) of the oligomer and the complementary DNA oligonucleotide.

The oligomer is preferably as defined above, where the at least one nucleoside analogue has the formula I where B is a nucleobase. Especially interesting are the cases where at least one nucleoside analogue includes a nucleobase selected from adenine and guanine.

Furthermore, with respect to specificity and affinity, the oligomer, when hybridised with a partially complementary DNA oligonucleotide, or a partially complementary RNA oligonucleotide, having one or more mismatches with said oligomer, should exhibit a reduction in T_m, as a result of said mismatches, which is equal to or greater than the reduction which would be observed with the corresponding unmodified reference oligonucleotide which does not comprise any nucleoside analogues. Also, the oligomer should have substantially the same sensitivity of T_m to the ionic strength of the hybridisation buffer as that of the corresponding unmodified reference oligonucleotide.

Oligomers defined herein are typically at least 1% modified, such as at least 2% modified, e.g. 3% modified, 4% modified, 5% modified, 6% modified, 7% modified, 8% modified, or 9% modified, at least 10% modified, such as at least 11% modified, e.g. 12% modified, 13% modified, 14% modified, or 15% modified, at least 20% modified, such as at least 30% modified, at least 50% modified, e.g. 70% modified, and in some interesting applications 100% modified.

The oligomers preferably have substantially higher 3'-exonucleolytic stability than the corresponding unmodified reference oligonucleotide.

It should be understood that oligomers (wherein LNAs are incorporated) and LNAs as such include possible salts

thereof, of which pharmaceutically acceptable salts are especially relevant. Salts include acid addition salts and basic salts. Examples of acid addition salts are hydrochloride salts, sodium salts, calcium salts, potassium salts, etc.. Examples of basic salts are salts where the (remaining) counter ion is selected from alkali metals, such as sodium and potassium, alkaline earth metals, such as calcium, and ammonium ions ($^+N(R^g)_3R^h$, where each of R^g and R^h independently designates optionally substituted C_{1-6}-alkyl, optionally substituted C_{2-6}-alkenyl, optionally substituted aryl, or optionally substituted heteroaryl). Pharmaceutically acceptable salts are, e.g., those described in Remington's Pharmaceutical Sciences, 17. Ed. Alfonso R. Gennaro (Ed.), Mack Publishing Company, Easton, Pa., U.S.A., 1985 and more recent editions and in Encyclopedia of Pharmaceutical Technology. Thus, the term "an acid addition salt or a basic salt thereof" used herein is intended to comprise such salts. Furthermore, the oligomers and LNAs as well as any intermediates or starting materials therefor may also be present in hydrate form.

DESCRIPTION OF THE FIGURES

FIG. 1.

Illustrates one embodiment of the invention.

FIG. 1-1.

Hybridization experiment performed with 3 different LNA modified oligos (see table 1-1) covalently immobilized to the wells of a microtiter-plate probe and a complementary target DNA oligo. The hybridisations were performed at variable concentrations of guanidine-hydrochloride (GnHCl) as indicated.

FIG. 2-1.

Competition assay illustrating the specificity of the LNA hybridisation performed at variable concentrations of guanidine hydrochloride. The amount of matching wild type oligo is kept constant at a concentration of 0.5 nM while the concentration of the competing (one base mismatching mutant oligo) is varied from 0.1 nM to 0.3 μM. The arrow indicates equimolar amounts of target and competing oligo. Also indicated is the hybridisation performance obtained in hybridisation buffers containing from 0 to 8 M guanidine hydrochloride (GnHCl).

FIG. 2-2.

Competition assay illustrating the specificity of the LNA hybridisation performed at variable concentrations of guanidine hydrochloride. The amount of matching mutant type oligo is kept constant at a concentration of 5 nM while the concentration of the competing (one base mismatching wild type oligo) is varied from 0.1 nM to 0.3 μM. The arrow indicates equimolar amounts of target and competing oligo. Also indicated is the hybridisation performance obtained in hybridisation buffers containing from 0 to 8 M guanidine hydrochloride (GnHCl).

FIG. 3-1.

LNA hybridisation experiment performed with 2 different LNA modified oligos (see table 3-1) covalently immobilized to the wells of a microtiter-plate probe and two complementary target DNA oligos. The hybridisations were performed at variable concentrations of guanidine thiocyanate (GnSCN) and in buffers based on either sodium citrate or phosphate as indicated.

FIG. 4-1.

Competition assay illustrating the specificity of the hybridisation performed phosphate based buffers and variable of guanidine thiocyanate (GnSCN). The amount of matching wild type oligo is kept constant at a concentration of 0.5 nM while the concentration of the competing (one base mismatching mutant oligo) is varied from 0.1 nM to 0.3 μM. The arrow indicates equimolar amounts of target and competing oligo. Also indicated is the hybridisation performance obtained in hybridisation buffers containing from 0 to 4 M GnSCN.

FIG. 4-2.

Competition assay illustrating the specificity of the hybridisation performed phosphate based buffers and variable of guanidine thiocyanate (GnSCN). The amount of matching mutant type oligo is kept constant at a concentration of 5 nM while the concentration of the competing (one base mismatching wild type oligo) is varied from 0.1 nM to 0.3 μM.

The arrow indicates equimolar amounts of target and competing oligo. Also indicated is the hybridisation performance obtained in hybridisation buffers containing from 0 to 4 M GnSCN.

FIG. 4-3.

Competition assay illustrating the specificity of the hybridisation performed sodium citrate based buffers and variable of guanidine thiocyanate (GnSCN). The amount of matching wild type oligo is kept constant at a concentration of 0.5 nM while the concentration of the competing (one base mismatching mutant oligo) is varied from 0.1 nM to 0.3 μM. The arrow indicates equimolar amounts of target and competing oligo. Also indicated is the hybridisation performance obtained in hybridisation buffers containing from 0 to 4 M guanidine thiocyanate (GnSCN).

FIG. 44.

Competition assay illustrating the specificity of the hybridisation performed sodium citrate based buffers and variable of guanidine thiocyanate (GnSCN). The amount of matching mutant type oligo is kept constant at a concentration of 5 nM while the concentration of the competing (one base mismatching wild type oligo) is varied from 0.1 nM to 0.3 μM. The arrow indicates equimolar amounts of target and competing oligo. Also indicated is the hybridisation performance obtained in in hybridisation buffers containing from 0 to 4 M GnSCN.

FIG. 6-1.

Detection of single nucleotide polymorphism. Biotinylated PCR amplicons from three human cell lines (HCV29, T112C1 and T112D1) and a plasmid were generated. All three human cell lines are wild type (G-allele) with respect to the ApoB3500 mutation. The "A-allele" plasmid contains the ApoB R3500Q mutation (a G->A transition at amino acid 3500 (arg->gln)). Each sample was tested against wild type (C8) or mutant (T8) specific LNA capture probes. Black bars: wild type. Light bars: mutant type.

FIG. 7-1.

Detection of plasmid DNA in bacterial cell lysates. Three different bacterial strains (see Table 7-1) were lysed and hybridised to either the C11 or the T11capture probes (see table 7-2) covalently immobilized to the wells of a microtiter-plate. The hybridisations were performed in a hybridisation buffer containing 2 M guanidine thiocyanate. NF1815 cells contain no plasmid, TOP10/pCR cells contain the pCR®2.1-TOPO plasmid without the ApoB sequence and TOP10/pApoBwt cells contain the ApoB3500 wild-type sequence inserted into the pCR®2.1-TOPO plasmid.

EXAMPLE 1

GnHCl Allows and Enhances LNA Hybridization in Phosphate Buffer

To study the effect that strong chaotrophic agents such as guanidine hydrochloride (GnHCl) exert on the hybridisation, the following experiment was carried out.

LNA modified oligos (see table 1-1) carrying a 5' anthraquinone were covalently immobilized to the wells of a microtiter-plate by UV irradiation and used as capture probes in a hybridisation assay with a complementary target DNA oligo. The hybrid was detected by including a 5' biotinylated DNA detection probe in the hybridisation mixture.

TABLE 1-1

Oligonucleotides studied

Name	EQ number	SEQ ID NO	Sequence	Characteristics
C8	EQ-3133	4	5'-AQ-tac atg tta tgc ttt GAC$^{met}\underline{C}^{met}$GT GTg-3'	5' anthraquinone modified LNA
C11	EQ-3131	5	5'-AQ-tac atg tta tgc ttt AAG AC$^{met}\underline{C}^{met}$ GTG TGc-3'	5' anthraquinone modified LNA
HEG C 8	EQ-3108	6	5'-AQ-HEG-AGA C$^{met}\underline{C}$G TGt-3'	5' hexaethylene glycol LNA
Wild type target molecule	EQ-3185	7	5'ttg aat tcc aag agc aca cgg tct tca gtg aag ctg cag ggc act tcc aa 3'	Wildtype, sense g/c pos. 9756 (50-mer)
Detection probe	EQ-3246	8	5'-biotin-ttg gaa gtg ccc tgc agc tt-3'	5' biotinylated DNA

Note:
LNA monomers are indicated in uppercase letters, DNA monomers are indicated in lowercase letters. C^{met} indicates that the monomer is 5-methyl cytosine LNA. 5'-AQ indicates that the oligo carries a 5'anthraquinone (AQ) and a C3 linker, the composition is AQ—CONH—$(CH_2)_3$-oligo. 5'-AQ—HEG indicates that the 5'end of the oligo is: AQ—CONH—$(CH_2)_3$—PO_4—$((CH_2)_2O)_5$—$(CH_2)_2$-oligo. 5'-biotin indicates that the 5'end of the oligo is: Biotin-$(CH_2)_4$—CONH—$(CH_2)_6$-oligo.

Immobilizing ApoB Capture Probes

Anthraquinone LNA capture probes (either C8, C11 or HEG C 8 —see table 1-1) were dissolved in 0.2 M NaCl at a concentration of 0.1 μM. 100 μL of the oligos were added to each of the wells of the microtiter-plate (C96 polysorp; Nalge Nunc International, Roskilde, Denmark), and exposed for 15 min. to soft UV light (approximately 350 nm) in a ULS-20-2 illuminator (UV-Lights Systems, Denmark) at maximal 35° C. The illuminator is equipped with 28 Philips Cleo Compact 25W-S light bulbs (14 located above and 14 located below the glass plate sample holder); only the upper bulbs were lit.

After incubation the wells were washed with first 300 μL of 0.4 M NaOH with 0.25% Tween 20 (Riedel-de Häen, Seeize, Germany) and then three times with 300 μL of deionized water.

Hybridization With Target and Detection Probe

Wild type (WT) target molecules (0.3 μM, EQ3185, SEQ ID NO 7) were added to micro-titer-plate wells coated with either C8, C11 or HEG C8 capture probe. The concentration of GnHCl in the hybridisation mixture was varied in two-fold "dilutions" as described below. The resulting concentrations of GnHCl were: 0.0078, 0.016, 0.032, 0.063, 0.13, 0.25, 0.5, 1, 2, 4 and 8 M.

Hybridization buffers were constructed by dissolving solid GnHCl to a final concentration of 8 M in 50 mM phosphate buffer, pH 7, with 0.1% Tween 20. Buffers with lower concentrations of GnHCl were the constructed by diluting the buffer containing 8 M GnHCl with a similar buffer containing 0 M GnHCl. 100 μL of hybridisation mix were added per microtiter-plate well. The capture and target oligos were allowed to hybridise for half an hour at 37° C. Then the wells were washed five times with 300 μL of 1xSSC with 0.1% Tween 20 (1xSSC is: 150 mM NaCl, 15 mM sodium citrate). Then, 100 μL of 0.12 μM detection probe (EQ-3246, SEQ ID NO 8) dissolved in 1xSSC with 0.1% Tween 20 were added and allowed to hybridise for half an hour at 37° C. Finally the microtiter-plate was washed three times with 1xSSC with 0.1% Tween 20.

The hybrids were detected by binding streptavidin-horseradish peroxidase (strA-HRP) to the biotinylated detection probe. The strA-HRP (Pierce, Rockford, Ill., U.S.A. Cat. no. 21126) was dissolved in 1xSSC with 0.1% Tween 20 at a concentration of 1 μg/mL. 100 μL was added per well and incubated 15 min. Then the plate was washed three times with 1xSSC; 0.1% Tween 20 and the signal developed in the OPD-assay.

OPD-assay

A master-mix containing: 6 mL of 0.1 M citrate buffer pH=5.0, two 2 mg ortho-phenylenediamine (OPD) tablets (Kem-En-Tech, Copenhagen, Denmark) and 2.5μL of 30% H_2O_2 was prepared. 100 μL of the master-mix is added to each reaction chamber and is left to incubate for one to 30 min. depending on enzyme activity. The assay is stopped with 100 μL of 0.5 M H_2SO_4 and the optical density is measured at λ=492 nm with an ELISA-reader.

Results

The results are shown in FIG. 1-1.

Conclusion

From the experiment it is concluded that it is possible to hybridise with good efficiency in buffers containing GnHCl. Surprisingly, it is observed that the hybridisation signal is enhanced with increasing concentrations of GnHCl. Even at very high (8 M) GnHCl concentrations, a high hybridisation signal is obtained. Actually, in the case of capture oligos with a DNA-linker (C8 (EQ-, SEQ ID NO 4) and C11 (EQ-3131, SEQ ID NO 5)) the hybridisation signal increases as a function of GnHCl concentration starting its effect at about 0.1 M and having its optimum from about 0.5 M to 5 M enhancing the hybridisation signal some 30%. Hybridization to capture probes immobilized to the surface of the microtiter-plate via the hexaethylene glycol linker (HEG C 8 (EQ-3108, SEQ ID NO 6)) appears less affected.

EXAMPLE 2.

Hybridization in GnHCl is Specific (Competition Experiment)

To study if hybridisation in buffers containing strong chaotrophic agents such as guanidine hydrochloride (GnHCl) can be performed at sufficiently high stringency to allow single-base discrimination, the following experiment was carried out.

LNA modified oligos (see table 2-1) carrying a 5' anthraquinone were covalently immobilized to the wells of a microtiter-plate by UV irradiation and used as capture probes in a hybridisation assay with a complementary target DNA oligo. The hybrid was detected by including a 5' biotinylated DNA detection probe in the hybridisation mixture.

TABLE 2-1

Oligonucleotides studied

Name	EQ number	SEQ ID NO	Sequence	Characteristics
C8	EQ-3133	4	5'-AQ-tac atg tta tgc ttt GAC^{met}C^{met}GT GTg-3'	5' anthraquinone modified LNA
T8	EQ-3134	9	5'-AQ-tac atg tta tgc ttt GAC^{met}TGT GTg-3'	5' anthraquinone modified LNA
Wild type target molecule	EQ-3185	7	5'ttg aat tcc aag agc aca cgg tct tca gtg aag ctg cag ggc act tcc aa 3'	Wildtype, sense g/c pos. 9756 (50-mer)
Mutation type target molecule	EQ-3187	10	5'ttg aat tcc aag agc aca cag tct tca gtg aag ctg cag ggc act tcc aa 3'	Sense a/t pos. 9756 (50-mer)
Detection probe	EQ-3246	8	5'-biotin-ttg gaa gtg ccc tgc agc tt-3'	5' biotinylated DNA

Note:
LNA monomers are indicated in uppercase letters, DNA monomers are indicated in lowercase letters. C^{met} indicates that the monomer is 5-methyl cytosine LNA. 5'-AQ indicates that the oligo carries a 5'anthraquinone (AQ) and a C3 linker, the composition is AQ—CONH—$(CH_2)_3$-oligo. 5'-biotin indicates that the 5'end of the oligo is: Biotin-$(CH_2)_4$—CONH—$(CH_2)_6$-oligo.

Immobilizing ApoB Capture Probes

Anthraquinone LNA capture probes (either C8 or T8—see table 2-1) were dissolved in 0.2 M NaCl at a concentration of 0.1 μM. 100 μL of the oligos were added to each of the wells of the microtiter-plate (C96 polysorp; Nalge Nunc International, Roskilde, Denmark), and exposed for 15 min. to soft UV light (approximately 350 nm) in a ULS-20-2 illuminator (UV-Lights Systems, Denmark) at maximal 35° C. The illuminator is equipped with 28 Philips Cleo Compact 25W-S light bulbs (14 located above and 14 located below the glass plate sample holder); only the upper bulbs were lit.

After incubation the wells were washed with first 300 μL of 0.4 M NaOH with 0.25% Tween 20 (Riedel-de Haen, Seeize, Germany) and then three times with 300 μL of deionized water.

Hybridization With Wild Type Target Molecule (EQ-3185) and Mutation Type Target Molecule (EQ-3187)

To wells coated with C8 capture probe (EQ 3133, SEQ ID NO 4) wild type target molecule (EQ3185, SEQ ID NO 7) was added in a constant low concentration (0.0005 μM) while the amount of the competing single base mismatching mutation type target molecule (EQ3187, SEQ ID NO 10) was varied in a five-fold "dilution series". The resulting concentrations of the mutation type target molecule were: 0.0001, 0.0005, 0.0025, 0.012, 0.06 and 0.30 μM.

To chambers coated with T8 capture probe (EQ3134, SEQ ID NO 9) mutation type target molecule was added in a constant low concentration (0.005 μM) while the amount of the competing single base mismatching wild type target molecule (EQ3185, SEQ ID NO 7) was varied in a five-fold "dilution series". The resulting concentrations of the wild type target molecule were: 0.0001, 0.0005, 0.0025, 0.012, 0.06 and 0.30 μM.

Hybridization buffers were constructed by dissolving solid GnHCl to a final concentration of 8 M in 50 μM phosphate buffer, pH 7, with 0.1% Tween 20. Buffers with lower concentrations of GnHCl were then constructed by diluting the buffer containing 8 M GnHCl with similar buffer containing 0 M GnHCl.

100 μL of hybridisation mix were added per microtiter-plate well. The capture and target oligos were allowed to hybridise for half an hour at 37° C. Then the wells were washed five times with 300 μL of 1xSSC with 0.1% Tween 20 (1xSSC is: 150 mM NaCl, 15 mM sodium citrate). Then, 100 μL of 0.12 μM detection probe (EQ-3246, SEQ ID NO 8) dissolved in 1xSSC with 0.1% Tween 20 were added and allowed to hybridise for half an hour at 37° C. Finally the microtiter-plate was washed three times with 1xSSC with 0.1% Tween 20.

The hybrids were detected by binding streptavidin-horseradish peroxidase to the biotinylated detection probe. The strA-HRP (Pierce, Rockford, Ill., U.S.A. Cat. no. 21126) was dissolved in 1xSSC with 0.1% Tween 20 at a concentration of 1 μg/mL. 100 μL was added per well and incubated 15 min. Then the plate was washed three times with 1xSSC; 0.1% Tween 20 and the signal developed in the OPD-assay.

OPD-assay

A master-mix containing: 6 mL of 0.1 M citrate buffer pH=5.0, two 2 mg ortho-phenylene-diamine (OPD) tablets (Kem-En-Tech, Copenhagen, Denmark) and 2.5 μL of 30% H_2O_2 was prepared. 100 μL of the master-mix is added to each reaction chamber and is left to incubate for one to 30 min. depending on enzyme activity. The assay is stopped with 100 μL of 0.5 M H_2SO_4 and the optical density is measured at λ=492 nm with an ELISA-reader.

Results:

The results are shown in FIGS. **2-1** and **2-2**.

Conclusion

Based on this experiment it is concluded that the stringency of the hybridisation—even at high concentrations of GnHCl—is compatible to the stringency observed in buffer without GnHCl.

With the C8 capture probe a clear increase in (non-specific) signal is not seen until the concentration of competing mutant type target molecule is above 0.05 μM. At this concentration the ratio of matching to mismatching oligo is 1:100. Equal contributions from the matching and the mismatching oligo (e.g. a two-fold increase in signal) is obtained at an amount of 0.2–0.5 μM competing oligo depending on the concentration of GnHCl. High stringency is seen at concentrations up to 4 M GnHCl. In these buffers equal signal is obtained at a ratio of matching to mismatching oligo of approximately 1:600. In other words, a signal to noise ratio of 1:600 is observed.

With the T8 capture probe a clear increase in (non-specific) signal is not seen until the concentration of competing wild type target molecule is above 0.1 μM. At this concentration the ratio of matching to mismatching oligo is 1:20. Equal contributions from the matching and the mismatching oligo (e.g. a two-fold increase in signal) are not obtained at any amount of competing oligo. Thus equal signal is assumed to be obtained at a ratio of matching to mismatching oligo that is higher than 1:60. In other words, a signal to noise ratio is observed that is higher than 1:60 (better than 1:100 extrapolated value).

It is concluded that hybridisation in buffers containing strong chaotrophic agents such as guanidine hydrochloride (GnHCl) can be performed at sufficiently high stringency to allow single-base discrimination.

EXAMPLE 3.

GnSCN Allows and Enhances Hybridization in Sodium Citrate and Phosphate Buffer

Standard lysis buffers for RNA preparation are often based on sodium citrate buffers, e.g.:

Glisin (1974) Biochemistry 13, 2633 and Chirwin (1979) Biochemistry 18, 5294. The following experiment was carried out to compare the hybridisation performance in guanidine thiocyanate (GnSCN) containing buffers based on either sodium citrate or phosphate.

LNA modified oligos (see table 3-1) carrying a 5' anthraquinone were covalently immobilized to the wells of a microtiter-plate by UV irradiation and used as capture probes in a hybridisation assay with a complementary target DNA oligo. The hybrid was detected by including a 5' biotinylated DNA detection probe in the hybridisation mixture.

TABLE 3-1

Oligonucleotides studied				
Name	EQ number	SEQ ID NO	Sequence	Characteristics
C8	EQ-3133	4	5'-AQ-tac atg tta tgc ttt GAC^{met} C^{met}GT GTg-3'	5' anthraquinone modified LNA
T8	EQ-3134	9	5'-AQ-tac atg tta tgc ttt GAC^{met} TGT GTg-3'	5' anthraquinone modified LNA
Wild type target molecule	EQ-3185	7	5'ttg aat tcc aag agc aca cgg tct tca gtg aag ctg cag ggc act tcc aa 3'	Wildtype, sense g/c pos. 9756 (50-mer)
Mutation type target molecule	EQ-3187	10	5'ttg aat tcc aag agc aca cag tct tca gtg aag ctg cag ggc act tcc aa 3'	Sense a/t pos. 9756 (50-mer)
Detection probe	EQ-3246	8	5'-biotin-ttg gaa gtg ccc tgc agc tt-3'	5' biotinylated DNA

Note:
LNA monomers are indicated in uppercase letters, DNA monomers are indicated in lowercase letters. C^{met} indicates that the monomer is 5-methyl cytosine LNA. 5'-AQ indicates that the oligo carries a 5'anthraquinone (AQ) and a C3 linker, the composition is AQ—CONH—$(CH_2)_3$-oligo. 5'-biotin indicates that the 5'end of the oligo is: Biotin-$(CH_2)_4$—CONH—$(CH_2)_6$-oligo.

Immobilizing ApoB Capture Probes

Anthraquinone LNA capture probes (either C8 or T8—see table 3-1) were dissolved in 0.2 M NaCl at a concentration of 0.1 μM. 100 μL of the oligos were added to each of the wells of the microtiter plate (C96 polysorp; Nalge Nunc International, Roskilde, Denmark), and exposed for 15 min. to soft UV light (approximately 350 nm) in a ULS-20-2 illuminator (UV-Lights Systems, Denmark) at maximal 35° C. The illuminator is equipped with 28 Philips Cleo Compact 25W-S light bulbs (14 located above and 14 located below the glass plate sample holder); only the upper bulbs were lit.

After incubation the wells were washed with first 300 μL of 0.4 M NaOH with 0.25% Tween 20 (Riedel-de Haën, Seelze, Germany) and then three times with 300 μL of deionized water.

Hybridization With WT (EQ-3185) and MUT (EQ-3187) Target Molecules

Wild type (WT) target molecules (0.012 μM) were added to microtiter-plate wells coated with C8 capture probe. The concentration of GnSCN in the hybridisation mixture was varied in two-fold "dilutions"—see below. The resulting concentrations of GnSCN were: 0.03, 0.06, 0.13, 0.25, 0.5, 1, 2 and 4 M.

Similarly, mutant type (MUT) target molecules (0.012 μM) were added to microtiter-plate wells coated with T8 capture probe and hybridised at 0.03, 0.06, 0.13, 0.25, 0.5, 1, 2 and 4 M GnSCN.

Hybridization buffers were constructed by dissolving solid GnSCN to a final concentration of 4 M either in 40 mM sodium citrate buffer, pH 7, with 0.5% Sarcosyl; or in 50 mM phosphate buffer, pH 7, with 0.1% Tween 20. Buffers with lower concentrations of GnSCN were then constructed by diluting the buffer containing 4 M GnSCN with a similar buffer containing 0 M GnSCN.

100 μL of hybridisation mix were added per microtiter-plate well. The capture and target oligos were allowed to hybridise for half an hour at 37° C. Then the wells were washed five times with 300 μL of 1xSSC with 0.1% Tween 20 (1xSSC is: 150 mM NaCl, 15 mM sodium citrate) Finally, 100 μL of 0.12 μM detection probe (EQ-3246, SEQ ID NO 8) in 1xSSC with 0.1% Tween 20 were added for half an hour at 37° C., and then washed three times with 1xSSC with 0.1% Tween 20.

The hybrids were detected by binding streptavidin-horseradish peroxidase to the biotinylated detection probe. The strA-HRP (Pierce, Rockford, Ill., USA. Cat. no. 21126) was dissolved in 1xSSC with 0.1% Tween 20 at a concentration of 1 μg/mL. 100 μL was added per well and incubated 15 min. Then the plate was washed three times with 1xSSC; 0.1% Tween 20 and the signal developed in the OPD-assay.

OPD-assay

A master-mix containing: 6 mL of 0.1 M citrate buffer pH=5.0, two 2 mg ortho-phenylenediamine (OPD) tablets (Kem-En-Tech, Copenhagen, Denmark) and 2.5 μL of 30% H_2O_2 was prepared. 100 μL of the master-mix is added to each reaction chamber and is left to incubate for one to 30 min. depending on enzyme activity. The assay is stopped with 100 μL of 0.5 M H_2SO_4 and the optical density is measured at λ=492 nm with an ELISA-reader.

Results

The results are shown in FIG. 3-1.

Conclusion

It is observed that the hybridisation signal is enhanced as a function of GnSCN concentration starting its effect at about 0.2 M and having its optimum from about 1 M to 2 M.

The most prominent enhancement by GnSCN is seen in the hybridisation between the C8 capture LNA probe and the WT target oligo in phosphate based buffers. At optimal concentration of GnSCN the signal was enhanced 75%.

The hybridisation with T8 and MUT oligo is less affected by GnSCN. In phosphate buffer high concentrations of GnSCN (4 M) had a negative effect.

In conclusion it is seen that buffers based on sodium citrate, e.g. the type that is used in many cell lysis-buffers, are at least as good as phosphate-based buffers, and that GnSCN, like guanidine hydrochloride (GnHCl), allows and indeed enhances hybridisation even at high concentrations.

EXAMPLE 4

Hybridization in GnSCN is Specific (Competition Experiment) Comparing Sodium Citrate and Phosphate Buffers

The following experiment was carried out to compare the hybridisation stringency in guanidine thiocyanate (GnSCN) containing buffers based on either sodium citrate or phosphate.

LNA modified oligos (see table 2-1) carrying a 5' anthraquinone were covalently immobilized to the wells of a microtiter-plate by UV irradiation and used as capture probes in a hybridisation assay with a complementary target DNA oligo. The hybrid was detected by including a 5' biotinylated DNA detection probe in the hybridisation mixture.

TABLE 4-1

Oligonucleotides studied				
Name	EQ number	SEQ ID NO	Sequence	Characteristics
C8	EQ-	4	5'-AQ-tac atg tta tgc ttt	5' anthraquinone

TABLE 4-1-continued

Oligonucleotides studied

Name	EQ number	SEQ ID NO	Sequence	Characteristics
	3133		GAC^{met}C^{met}GT GTg-3'	modified LNA
T8	EQ-3134	9	5'-AQ-tac atg tta tgc ttt GAC^{met} TGT GTg-3'	5' anthraquinone modified LNA
Wild type target molecule	EQ-3185	7	5'ttg aat tcc aag agc aca cgg tct tca gtg aag ctg cag ggc act tcc aa 3'	Wildtype, sense g/c pos. 9756 (50-mer)
Mutation type target molecule	EQ-3187	10	5'ttg aat tcc aag agc aca cag tct tca gtg aag ctg cag ggc act tcc aa 3'	Sense a/t pos. 9756 (50-mer)
Detection probe	EQ-3246	8	5'-biotin-ttg gaa gtg ccc tgc agc tt-3'	5' biotinylated DNA

Note:
LNA monomers are indicated in uppercase letters, DNA monomers are indicated in lowercase letters. C^{met} indicates that the monomer is 5-methyl cytosine LNA. 5'-AQ indicates that the oligo carries a 5'anthraquinone (AQ) and a C3 linker, the composition is AQ—CONH—$(CH_2)_3$-oligo. 5'-biotin indicates that the 5'end of the oligo is: Biotin-$(CH_2)_4$—CONH—$(CH_2)_6$-oligo.

Immobilizing ApoB Capture Probes

Anthraquinone LNA capture probes (either C8 or T8—see table 4-1) were dissolved in 0.2 M NaCl at a concentration of 0.1 μM. 100 μL of the oligos were added to each of the wells of the microtiter-plate (C96 polysorp; Nalge Nunc International, Roskilde, Denmark), and exposed for 15 min. to soft UV light (approximately 350 nm) in a ULS-20-2 illuminator (UV-Lights Systems, Denmark) at maximal 35° C. The illuminator is equipped with 28 Philips Cleo Compact 25W-S light bulbs (14 located above and 14 located below the glass plate sample holder); only the upper bulbs were lit.

After incubation the wells were washed with first 300 μL of 0.4 M NaOH with 0.25% Tween 20 (Riedel-de Häen, Seelze, Germany) and then three times with 300 μL of deionized water.

Hybridization With Wild Type Target Molecule (EQ-3185) and Mutation Type Target Molecule (EQ-3187)

To wells coated with C8 capture probe (EQ 3133, SEQ ID NO 4) wild type target molecule (EQ3185, SEQ ID NO 7) was added in a constant low concentration (0.0005 μM) while the amount of the competing single base mismatching mutation type target molecule (EQ3187, SEQ ID NO 10) was varied in a five-fold "dilution series". The resulting concentrations of the mutation type target molecule were: 0.0001, 0.0005, 0.0025, 0.012, 0.06 and 0.30 μM.

To chambers coated with T8 capture probe (EQ3134, SEQ ID NO 9) mutation type target molecule was added in a constant low concentration (0.005 μM) while the amount of the competing single base mismatching wild type target molecule (EQ3185, SEQ ID NO 7) was varied in a five-fold "dilution series". The resulting concentrations of the wild type target molecule were: 0.0001, 0.0005, 0.0025, 0.012, 0.06 and 0.30 μM.

Hybridization buffers were constructed by dissolving solid GnSCN to a final concentration of 4 M either in 40 mM sodium citrate buffer, pH 7, with 0.5% Sarcosyl; or in 50 μM phosphate buffer, pH 7, with 0.1% Tween 20. Buffers with lower concentrations of GnSCN were then constructed by diluting the buffer containing 4 M GnSCN with a similar buffer containing 0 M GnSCN.

100 μL of hybridisation mix were added per microtiter-plate well. The capture and target oligos were allowed to hybridise for half an hour at 37° C. Then the wells were washed five times with 300 μL of 1×SSC with 0.1% Tween 20 (1×SSC is: 150 mM NaCl, 15 mM sodium citrate). Then, 100 μL of 0.12 μM detection probe (EQ-3246, SEQ ID NO 8) dissolved in 1×SSC with 0.1% Tween 20 were added and allowed to hybridise for half an hour at 37° C. Finally the microtiter-plate was washed three times with 1×SSC with 0.1% Tween 20.

The hybrids were detected by binding streptavidin-horseradish peroxidase to the biotinylated detection probe. The strA-HRP (Pierce, Rockford, Ill., USA. Cat. no. 21126) was dissolved in 1×SSC with 0.1% Tween 20 at a concentration of 1 μg/mL. 100 μL was added per well and incubated 15 min. Then the plate was washed three times with 1×SSC; 0.1% Tween 20 and the signal developed in the OPD-assay.

OPD-assay

A master-mix containing: 6 mL of 0.1 M citrate buffer pH=5.0, two 2 mg ortho-phenylenediamine (OPD) tablets (Kem-En-Tech, Copenhagen, Denmark) and 2.5 μL of 30% H_2O_2 was prepared. 100 μL of the master-mix is added to each reaction chamber and is left to incubate for one to 30 min. depending on enzyme activity. The assay is stopped with 100 μL of 0.5 M H_2SO_4 and the optical density is measured at λ=492 nm with an ELISA-reader.

Results

The results are shown in FIGS. 4-1, 4-2, 4-3 and 4-4.

Conclusion

Based on this experiment it is concluded that the stringency of the hybridisation—even at high concentrations of GnSCN—is compatible to the stringency observed in buffer without GnSCN. It is furthermore concluded that the stringency is comparable in sodium citrate- and phosphate-based buffers.

With the C8 capture probe a clear increase in (non-specific) signal is not seen until the concentration of competing mutant type target molecule is above 0.05 μM. At this concentration the ratio of matching to mismatching oligo is 1:100. Equal contributions from the matching and the mismatching oligo (e.g. a two-fold increase in signal) is, with the exception of C8 hybridisation in 4 M GnSCN, PO_4-buffer, not obtained at any amount of competing oligo. In the case of the hybridisation to C8 in 4 M GnSCN, PO_4 equal signal is obtained at approximately 0.15 μM indicating a signal to noise ratio of approximately 300. In the remaining hybridisations the signal to noise ratio is better than 1:600.

With the T8 capture probe a clear increase in (non-specific) signal is not seen until the concentration of competing wild type target molecule is over 0.1 μM. At this concentration the ratio of matching to mismatching oligo is 1:20. Equal contributions from the matching and the mismatching oligo (e.g. a two-fold increase in signal) is not obtained at any amount of competing oligo. Thus equal signal is assumed to be obtained at a ratio of matching to mismatching oligo that is higher than 1:60. In other words, a signal to noise ratio that is higher than 1:60 is observed.

The hybridisation stringency is almost the same in buffers based on either sodium citrate or phosphate. However, higher hybridisation signals were obtained in sodium citrate based buffers.

It is concluded that hybridisation in buffers containing strong chaotrophic agents such as in guanidine thiocyanate (GnSCN) can be performed at sufficiently high stringency to allow detection of single base differences and that buffers based on either sodium citrate or phosphate can be used.

EXAMPLE 5

Thermostability of DNA and LNA Oligonucleotides in Buffers Containing Guanidine Hydrochloride

The thermostability of the all-DNA and LNA modified oligonucleotides was determined spectrophotometrically

using a spectrophotometer equipped with a thermoregulated Peltier element (Perkin Elmer, UV Lambda 40). Hybridization mixtures of 1 ml were prepared containing either of 2 different buffers (4 M GnHCl in 50 mM Na—PO_4, pH 6.8, 0.1 mM EDTA; or 50 mM Na—PO_4, pH 6.8, 115 mM NaCl, 0.1 mM EDTA) and equimolar (1 μM) amounts of the LNA modified oligonucleotides and their complementary or mismatched DNA oligonucleotides. Identical hybridisation mixtures using the unmodified oligonucleotides were prepared as references.

Each sample was heated in an eppendorf tube to 90° C. in a heating block and allowed to cool slowly within the turned off heating block to room temperature. The sample was transferred into a 500 μL quartz cuvette (Perkin Elmer). Samples were then measured on the UV Lambda 40 spectrophotometer. The samples were measured at 260 nm while the temperature was raised with 1° C. per minute. The T_m's were obtained as the first derivative of the melting curves. Table 5-2 summarizes the results. Table 5-1 summarizes the oligos used.

TABLE 5-1

Oligonucleotides studied

Name	EQ number	SEQ ID NO	Sequence	Characteristics
112T	EQ-3485	11	5'-$C^{met}GC^{met}$ $AC^{met}A$ $C^{met}Gt$-3'	LNA
as112t	EQ-3493	12	5'-acg tgt gcg-3'	LNA
as112c	EQ-3495	13	5'-acg tgc gcg-3'	all-DNA
158T	EQ-3489	14	5'-GGC^{met} $AC^{met}T$ $TC^{met}t$-3'	LNA
as158t	EQ-3497	15	5'-aga agt gcc-3'	LNA
as158c	EQ-3499	16	5'-aga agc gcc-3'	all-DNA

Note:
LNA monomers are indicated in uppercase letters, DNA monomers are indicated in lowercase letters. C^{met} indicates that the monomer is 5-methyl cytosine LNA.

Results

Table 5-2 shows the thermostability of DNA and LNA oligonucleotides in buffers containing guanidine hydrochloride.

TABLE 5-2

		T_m in hybridisation buffer containing	
oligo pair	match	165 mM Na^+	4 M GnHCl
LNA (EQ3485):DNA (EQ3493)	perfect match	73.8° C.	65.4° C.
LNA (EQ3485):DNA (EQ3495)	one mismatch	51.5° C. (ΔT_m 22.3° C.)	41.1° C. (ΔT_m 24.3° C.)
LNA (EQ3489):DNA (EQ3497)	perfect match	69.9° C.	61.2° C.
LNA (EQ3489):DNA (EQ3499)	one mismatch	47.0° C. (ΔT_m 22.9° C.)	33.2° C. (ΔT_m 28.0° C.)

Note:
ΔT_m indicates the difference in T_m between the perfect matching LNA:DNA hetero-duplex and the duplex in question.

Conclusion

From the experiment it is concluded that hybridisation occurs in buffers containing high concentrations of strong chaotrophic agents.

When a single mismatch is introduced into the target DNA oligonucleotides, the T_m of LNA:DNA heteroduplexes drops significantly. The results indicate that the T_m change is more pronounced in guanidine containing buffers compared to standard buffers, suggesting that hybridisation buffers containing strong chaotrophic agents may be advantageous for single base discrimination by hybridisation.

EXAMPLE 6

Detection of Single Base Polymorphism by Hybridisation of PCR Products to LNA Capture Oligos

As a first step to test if it is possible to detect a specific sequence in a complex biological sample, the following experiment was carried out.

The product of the PCR is considerably more complex than adding synthetic 50'mer template, and this may challenge the hybridisation process. To test if hybridisation in chaotrophic buffers was sufficiently discriminatory and sensitive, PCR reactions were performed on various templates containing either wild type or mutant type ApoB3500, and tested in a LNA hybridisation assay. The assay consisted of 5' anthraquinone immobilized LNA modified oligos (see table 6-1) functioning as capture probe and the PCR product as complementary target DNA oligo. The hybrid was detected by binding streptavidin-horseradish peroxidase to the biotinylated end of the PCR product and afterwards performing an OPD assay.

TABLE 6-1

Oligonucleotides applied

Name	EQ number	SEQ ID NO	Sequence	Characteristics
C8	EQ-3133	4	5'-AQ-tac atg tta tgc ttt GAC^{met} $\underline{C}^{met}GT$ GTg-3'	5' anthraquinone modified LNA
T8	EQ-3134	9	5'-AQ-tac atg tta tgc ttt GAC^{met} $\underline{T}GT$ GTg-3'	5' anthraquinone modified LNA
Forward primer	EQ-3198	17	5'-biotin-cta gtg agg cca aca ctt act tga att cca aga gc-3'	5' biotinylated DNA primer, sense (35-mer)
Backward primer	EQ-3213	3	5'-gtt ttt cgt act gtg ctc cca gag-3'	DNA primer, sense (24-mer)

Note:
LNA monomers are indicated in uppercase letters, DNA monomers are indicated in lowercase letters. C^{met} indicates that the monomer is 5-methyl cytosine LNA. 5'-AQ indicates that the oligo carries a 5' anthraquinone (AQ) and a C3 linker, the composition is AQ—CONH—$(CH_2)_3$-oligo. 5'-biotin indicates that the 5'end of the oligo is: Biotin-$(CH_2)_4$—CONH—$(CH_2)_6$-oligo.

TABLE 6-2

PCR template

Sample	Source	Characteristics
HCV29	Human DNA	ApoB35000, wild type, sense g/c pos. 9756
T112C1	Human DNA	ApoB35000, wild type, sense g/c pos. 9756
T112D1	Human DNA	ApoB35000, wild type, sense g/c pos. 9756
"A-allele"-plasmid	Plasmid	ApoB35000, mutant type, sense a/t pos. 9756

Note:
With the forward and backward primers applied on these total DNA or plasmid preparations the expected PCR amplified fragment is 167 bp long and will be 5' biotinylated on the sense strand.

Synthesis and Analysis of Primers

DNA primers were obtained as HPLC purified oligos from a commercial source (DNA Technology, Aarhus, Denmark).

Sample Preparation

1) Human genomic DNA:

Human genomic DNA was isolated by standard phenol extraction (Sambrook et al. (1989) Molecular Cloning, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.) from 3 different human cancer cell lines: HCV29, T 112C1, T 112D1 (Skouv et al. (1989) Mol Carcin. 2, 59–62) that are wildtype with respect to the ApoB3500 polymorphism (Ludwig et al. (1987) DNA 6: 363–372; accession no. M 19828).

2) "A-allele"-plasmid:

Human genomic DNA was isolated from 5 ml of whole blood, using the DNA Isolation Kit for Mammalian Blood (Roche Molecular Systems cat. no. 1 667 327. Roche Molecular Biochemicals, Hvidovre, Denmark) and closely following the recommendations of the manufacturer.

The "A-allele" of the ApoB gene was generated by PCR-amplification of part of the wild type human ApoB gene comprising the ApoB3500 locus. The PCR fragment was cloned into plasmid pCR®2.1-TOPO using the TOPO™ TA Cloning® Kit (Invitrogen, cat. no. K4500-01, Invitrogen Corporation, Carlsbad Calif. USA). Plasmid DNA was purified from bacterial cultures, using QUIAGEN® Plasmid Kits (QIAGEN GmbH, Hilden, Germany). The DNA sequence of the insert was verified by DNA sequencing using an ALFexpress II DNA Analysis System (Amersham Pharmacia Biotech) and closely following the recommendations of the manufacturer.

PCR on Sample Preparations

PCR master-mix for 6 reactions:

148.50 μL H_2O

30 μL 10×AmpliTaq Gold buffer (Perkin-Elmer Corporation, Norwalk, Conn., USA).

18 μL $MgCl_2$ (25 mM)

30 μL dNTP (2 mM)

30 μL forward primer EQ3198 (SEQ ID NO 17) (10 μM)

30 μL backward primer EQ3213 (SEQ ID NO 3) (10 μM)

1.5 μL AmpliTaq Gold® DNA Polymerase (5 U/μL) (Perkin Elmer cat. no. N808-0240, Perkin-Elmer Corporation, Norwalk, Conn., USA).

PCR Amplification

The PCR reactions were carried out in 0.5 mL thin-wall tubes using an Eppendorf Mastercycler Gradient thermocycler (Eppendorf—Netheler—Hinz GmbH, Hamburg, Germany). To 48 μL master-mix 2 μL sample were added.

Termocycling

Denaturation: 94° C., 15 min.

Amplification (30 cycles): 94° C., 40 sec.; 56° C., 40 sec.; 72° C., 40 sec.

Elongation: 72° C., 10 min.

Termination: 4° C., ∞.

Detection

The PCR products were subsequently analyzed by standard gel electrophoresis on a 2% agarose gel (LE, Analytical Grade; Promega Corporation, Madison, USA) including Gel-Star® (FMC BioProducts, Rockland, Me., USA) diluted 1:30.000 in the gel and 1×Tris-acetate/EDTA electrophoresis buffer (0.04 M Tris-acetate; 0.001 M EDTA). To 5 μL of each PCR reaction 1 μL of 6×loading buffer (40% sucrose, 0.25% bromophenol blue, 0.25% xylene cyanol, 0.1 M EDTA pH 8.0) was added). The gel was run for approximately 1 h at a constant voltage of 7 V/cm.

For permanent record the gel was photographed by standard Polaroid (Polaroid LTD., St. Albans, UK) photography using an appropriate UV-transilluminator (Model TM-20E UV Products, Upland, Calif., USA) and filter (Kodak Wratten #9 Eastman Kodak Co., Rochester, N.Y., USA).

Immobilizing ApoB Capture Probes

Anthraquinone LNA capture probes (either C8 or T8—see table 6-1) were dissolved in 0.2 M NaCl at a concentration of 0.1 μM. 100 μL of the oligos were added to each of the wells of the microtiter-plate (C96 polysorp; Nalge Nunc International, Roskilde, Denmark), and exposed for 15 min. to soft UV light (approximately 350 nm) in a ULS-20-2 illuminator (UV-Lights Systems, Denmark) at a maximal temperature of 35° C. The illuminator is equipped with 28 Philips Cleo Compact 25W-S light bulbs (14 located above and 14 located below the glass plate sample holder); only the upper bulbs were lit.

After incubation the wells were washed with first 300 μL of 0.4 M NaOH with 0.25% Tween 20 (Riedel-de Haën, Seelze, Germany) and then three times with 300 μL of deionized water.

Hybridisation With PCR Reaction

To chambers with immobilized C8 or T8 capture probe 5 μL of PCR reaction together with 95 μL of 2 M GnSCN sodium citrate buffer were added and left to incubate for half an hour at 37° C.

Hybridisation buffers were 2 M GnSCN in 40 mM sodium citrate buffer, pH 7, with 0.5% Sarcosyl.

After hybridisation the wells were washed five times with 300 μL of 1×SSC with 0.1% Tween 20 (1×SSC is: 150 mM NaCl, 15 mM sodium citrate).

The hybrids were detected by binding streptavidin-horseradish peroxidase to the biotinylated PCR product. The strA-HRP (Pierce, Rockford, Ill., USA. Cat. no. 21126) was dissolved in 1×SSC with 0.1% Tween 20 at a concentration of 1 μg/mL. 100 μL was added per well and incubated 15 min. Then the plate was washed three times with 1×SSC; 0.1% Tween 20 and the signal developed in the OPD-assay.

OPD-assay

A master-mix containing: 6 mL of 0.1 M citrate buffer pH=5.0, two 2 mg ortho-phenylenediamine (OPD) tablets (Kem-En-Tech, Copenhagen, Denmark) and 2.5 μL of 30% H_2O_2 as prepared. 100 μL of the master-mix is added to each reaction chamber and is left to incubate for one to 30 min. depending on enzyme activity. The assay is stopped with 100 μL of 0.5 M H_2SO_4 and the optical density is measured at λ=492 nm with an ELISA-reader.

Results

The results are shown in FIG. 6-1.

Conclusion

In all four PCR reactions it is possible to detect single base differences by hybridisation in 2 M GnSCN. As seen also in the previous examples the signal for "A-allele" capture probe (T8) is lower than the signal obtained with the C8 LNA capture probe, yet the signal is very clear also in this case, indicating that hybridisation in 2 M GnSCN is highly stringent.

EXAMPLE 7

Detection of Plasmid DNA in Bacterial Cell Lysate

To study if the hybridisation and extraction methods of the present invention may be applied to a complex biological mixture of nucleic acid and non-nucleic acid, the following experiment was carried out.

Briefly, three different strains of bacteria (*E. coli* K12), two plasmid-containing and one strain without plasmid (see table 7-1), were cultured, lysed, and the plasmid was detected by hybridisation in a guanidine thiocyanate (GnSCN) containing buffer.

TABLE 7-1

Bacterial Strains.		
strain name	genotype	reference
NF1815	MC1000 recA1	Casadaban (1980) J. Mol. Biol. 138, 179–207.
TOP10/ pCR	F⁻mcrA_(mrr-hsdRMS-mcrBC) _80lacZ_M15_lac_74 recA1 deoR araD139_(ara-leu) 7697 galU galK rpsL (Str^R) endA1 nupG/pCR ®2.1-TOPO	Invitrogen, cat. no. K4500-01, Invitrogen Corporation, Carlsbad CA USA
TOP10/ pApoBwt	F⁻mcrA_(mrr-hsdRMS-mcrBC) _80lacZ_M15_lac_74 recA1 deoR araD139_(ara-leu) 7697 galU galK rpsL (Str^R) endA1 nupG/pApoBwt	

Cloning of ApoBwt Plasmid

Human genomic DNA was isolated from 5 ml of whole blood, using the DNA Isolation Kit for Mammalian Blood (Roche Molecular Systems cat. no. 1 667 327. Roche Molecular Biochemicals, Hvidovre, Denmark) and closely following the recommendations of the manufacturer.

The "G-allele" of the ApoB gene was generated by PCR-amplification of part of the wild type human ApoB gene comprising the ApoB3500 locus (Ludwig et al. (1987) DNA 6: 363–372; accession no. M19828, SEQ ID NO 1 and SEQ ID NO 2). The PCR fragment was cloned into plasmid pCR®2.1-TOPO using the TOPO™ TA Cloning® Kit (Invitrogen, cat. no. K4500-01, Invitrogen Corporation, Carlsbad Calif. USA). Plasmid DNA was purified from bacterial cultures, using QUIAGEN® Plasmid Kits (QIAGEN GmbH, Hilden, Germany). The DNA sequence of the insert was verified by DNA sequencing using an ALFexpress II DNA Analysis System (Amersham Pharmacia Biotech) and closely following the recommendations of the manufacturer. The resulting plasmid was named pApoBwt.

The TOP10/pCR strain contains the pCR®2.1-TOPO plasmid without any ApoB insert.

Preparation of Bacterial Cell Lysate

Bacteria were grown in LB media (Sambrook et al. (1989) Molecular Cloning, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.) with 100 μg/mL ampicillin overnight at 37° C. on a shaking device.

The next day the cells were centrifuged (15 min, 8000 r/min) and then resuspended in 1/50 volume 50 mM Tris-Cl (pH 8). The cells were then either sonicated for 45 sec. on ice or added 0.250 mL 100 mg/mL lysozyme for every 2.5 mL resuspended celles and left to incubate for 15 min. at room temperature. The lysed cells were then kept at −20° C.

Detection

LNA modified oligos (see table 7-2) carrying a 5' anthraquinone were covalently immobilized to the wells of a microtiter-plate by UV irradiation and used as capture probes in a hybridisation assay with a complementary target DNA oligo. The hybrid was detected by including a 5' biotinylated DNA detection probe in the post-hybridisation mixture.

TABLE 7-2

Oligonucleotides studied				
Name	EQ number	SEQ ID NO	Sequence	Characteristics
C11	EQ-3131	5	5'-AQ-tac atg tta tgc ttt AAG AC$^{met}\underline{C}^{met}$GTG TGc-3'	5' anthraquinone modified LNA
T11	EQ-3132	18	5'-AQ-tac atg tta tgc ttt AAG AC$^{met}\underline{T}$ GTG TGc-3'	5' anthraquinone modified LNA
Detection probe	EQ-3246	8	5'-biotin-ttg gaa gtg ccc tgc agc tt-3'	5' biotinylated DNA

Note:
LNA monomers are indicated in uppercase letters, DNA monomers are indicated in lowercase letters. C^{met} indicates that the monomer is 5-methyl cytosine LNA. 5'-AQ indicates that the oligo carries a 5'anthraquinone (AQ) and a C3 linker, the composition is AQ—CONH—$(CH_2)_3$-oligo. 5'-biotin indicates that the 5'end of the oligo is: Biotin-$(CH_2)_4$—CONH—$(CH_2)_6$-oligo.

Immobilizing ApoB Capture Probes

Anthraquinone LNA capture probes (C11, T11—see table 7-1) were dissolved in 0.2 M NaCl at a concentration of 0.1 μM. 100 μL of the oligos were added to each of the wells of the microtiter plate (C96 polysorp; Nalge Nunc International, Roskilde, Denmark), and exposed for 15 min. to soft UV light (approximately 350 nm) in a ULS-20-2 illuminator (UV-Lights Systems, Denmark) at maximal 35° C. The illuminator is equipped with 28 Philips Cleo Compact 25W-S light bulbs (14 located above and 14 located below the glass plate sample holder); only the upper bulbs were lit.

After incubation the wells were washed with first 300 μL of 0.4 M NaOH with 0.25% Tween 20 (Riedel-de Häen, Seelze, Germany) and then three times with 300 μL of deionized water.

Hybridization

50 μL of the bacterial cell lysate was mixed with 50 μL (4 M GnSCN, 25 mM sodium citrate, pH 7, 0.5% Sarcosyl) and added to microtiter-plate wells coated with either C8 or T8 LNA capture probe. The mixture was allowed to incubate overnight at 37° C.

Then the wells were washed five times with 300 μL of 1×SSC with 0.1% Tween 20 (1×SSC is: 150 mM NaCl, 15 mM sodium citrate). 100 μL of 0.12 μM detection probe (EQ-3246, SEQ ID NO 8) in 1×SSC with 0.1% Tween 20 were added and allowed to hybridise overnight at 37° C., and then washed three times with 1×SSC with 0.1% Tween 20.

The hybrids were detected by binding streptavidin-horseradish peroxidase to the biotinylated detection probe. The strA-HRP (Pierce, Rockford, Ill., USA. Cat. no. 21126) was dissolved in 1×SSC with 0.1% Tween 20 at a concentration of 1 μg/mL. 100 μL was added per well and incubated 15 min. Then the plate was washed three times with 1×SC; 0.1% Tween 20 and the signal developed in the OPD-assay.

OPD-assay

A master-mix containing: 6 mL of 0.1 M citrate buffer pH=5.0, two 2 mg ortho-phenylenediamine (OPD) tablets (Kem-En-Tech, Copenhagen, Denmark) and 2.5 μL 30% H_2O_2 was prepared. 100 μL of the master-mix is added to each reaction chamber and is left to incubate for one to 30 min. depending on enzyme activity. The assay is stopped with 100 μL of 0.5 M H_2SO_4 and the optical density is measured at λ=492 nm with an ELISA-reader.

Results

The results are shown in FIG. 7-1.

Conclusion

From the results it is concluded that the pApoBwt plasmid can be captured and detected both in bacteria lysed by sonication and lysozyme treatment. Since plasmids are double-stranded, supercoiled DNA molecules, this experiment indicates that it is possible to detect double-stranded, supercoiled DNA molecules in a complex biological sample such as a crude bacterial lysate.

```
                    SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 18

<210> SEQ ID NO 1
<211> LENGTH: 50
<212> TYPE: DNA
<213> ORGANISM: Homo Sapiens

<400> SEQUENCE: 1

cttacttgaa ttccaagagc acacggtctt cagtgaagct gcagggcact                50


<210> SEQ ID NO 2
<211> LENGTH: 50
<212> TYPE: DNA
<213> ORGANISM: Homo Sapiens

<400> SEQUENCE: 2

cttacttgaa ttccaagagc acacagtctt cagtgaagct gcagggcact                50


<210> SEQ ID NO 3
<211> LENGTH: 24
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic primer

<400> SEQUENCE: 3

gtttttcgta ctgtgctccc agag                                            24


<210> SEQ ID NO 4
<211> LENGTH: 24
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic primer
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (16)...(23)
<223> OTHER INFORMATION: LNA
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (18)...(19)
<223> OTHER INFORMATION: 5-methyl-cytosine LNA

<400> SEQUENCE: 4

tacatgttat gctttgaccg tgtg                                            24


<210> SEQ ID NO 5
<211> LENGTH: 27
<212> TYPE: DNA
<213> ORGANISM: SyArtificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthethic primer
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (16)...(26)
<223> OTHER INFORMATION: LNA
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (20)...(21)
<223> OTHER INFORMATION: 5-methyl-cytosine LNA
```

<400> SEQUENCE: 5

tacatgttat gctttaagac cgtgtgc 27

<210> SEQ ID NO 6
<211> LENGTH: 9
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic primer
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)...(9)
<223> OTHER INFORMATION: LNA
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)...(1)
<223> OTHER INFORMATION: 5-hexaethylenglycol LNA
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (4)...(4)
<223> OTHER INFORMATION: 5-methyl-cytosine LNA

<400> SEQUENCE: 6

agaccgtgt 9

<210> SEQ ID NO 7
<211> LENGTH: 50
<212> TYPE: DNA
<213> ORGANISM: Homo Sapiens

<400> SEQUENCE: 7

ttgaattcca agagcacacg gtcttcagtg aagctgcagg gcacttccaa 50

<210> SEQ ID NO 8
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic primer

<400> SEQUENCE: 8

ttggaagtgc cctgcagctt 20

<210> SEQ ID NO 9
<211> LENGTH: 24
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic primer
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (16)...(23)
<223> OTHER INFORMATION: LNA
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (18)...(18)
<223> OTHER INFORMATION: 5-methyl-cytosine LNA

<400> SEQUENCE: 9

tacatgttat gctttgactg tgtg 24

<210> SEQ ID NO 10
<211> LENGTH: 50
<212> TYPE: DNA
<213> ORGANISM: Homo Sapiens

<400> SEQUENCE: 10

ttgaattcca agagcacaca gtcttcagtg aagctgcagg gcacttccaa 50

<210> SEQ ID NO 11
<211> LENGTH: 9
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthethic primer
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)...(8)
<223> OTHER INFORMATION: LNA
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)...(1)
<223> OTHER INFORMATION: 5-methyl-cytosine LNA
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (3)...(3)
<223> OTHER INFORMATION: 5-methyl-cytosine LNA
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (5)...(5)
<223> OTHER INFORMATION: 5-methyl-cytosine LNA
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (7)...(7)
<223> OTHER INFORMATION: 5-methyl-cytosine LNA

<400> SEQUENCE: 11

cgcacacgt 9

<210> SEQ ID NO 12
<211> LENGTH: 9
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic primer

<400> SEQUENCE: 12

acgtgtgcg 9

<210> SEQ ID NO 13
<211> LENGTH: 9
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic primer

<400> SEQUENCE: 13

acgtgcgcg 9

<210> SEQ ID NO 14
<211> LENGTH: 9
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic primer
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)...(8)
<223> OTHER INFORMATION: LNA
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (3)...(3)
<223> OTHER INFORMATION: 5-methyl-cytosine LNA
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (5)...(5)
<223> OTHER INFORMATION: 5-methyl-cytosine LNA
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (8)...(8)

-continued

```
<223> OTHER INFORMATION: 5-methyl-cytosine LNA

<400> SEQUENCE: 14

ggcacttct                                                    9


<210> SEQ ID NO 15
<211> LENGTH: 9
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic primer

<400> SEQUENCE: 15

agaagtgcc                                                    9


<210> SEQ ID NO 16
<211> LENGTH: 9
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic primer

<400> SEQUENCE: 16

agaagcgcc                                                    9


<210> SEQ ID NO 17
<211> LENGTH: 35
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic primer

<400> SEQUENCE: 17

ctagtgaggc caacacttac ttgaattcca agagc                      35


<210> SEQ ID NO 18
<211> LENGTH: 27
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic primer
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (16)...(26)
<223> OTHER INFORMATION: LNA
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (20)...(20)
<223> OTHER INFORMATION: 5-methyl-cytosine LNA

<400> SEQUENCE: 18

tacatgttat gctttaagac tgtgtgc                                27
```

What is claimed is:

1. A method for isolating a target nucleic acid comprising

a) providing a sample containing nucleic acids,

b) treating the sample with a lysing buffer containing a chaotropic agent to lyse cellular material in the sample, dissolve the components and denature the nucleic acids in the sample,

c) contacting the nucleic acids released from the sample with at least one capturing LNA-probe, said capturing probe being substantially complementary to the target nucleic acid.

2. A method according to claim 1, wherein the capturing LNA-probe is covalently attached to a ligand.

3. A method according to claim 1, wherein the capturing LNA-probe is covalently attached to a solid surface.

4. A method according to claim 2, wherein the ligand covalently attached to the LNA-probe is coupled to an anti-ligand, said anti-ligand being covalently attached to a solid surface.

5. A method according to claims 3 or 4, wherein after the contact of the nucleic acids released from the sample with the LNA attached to the solid surface, the solid surface is separated from excess material.

6. A method according to claim 5, wherein the solid surface is washed with buffer to remove excess material.

7. A method according to claim 1 wherein the capturing probe is complementary to the target nucleic acid.

8. A method according to claim 1 wherein the capturing probe is between 4 and 50 nucleotides long.

9. A method according to claim 1 wherein the capturing probe is between 8 and 30 nucleotides long.

10. A method according to claim 1 wherein the capturing probe is between 8 and 20 nucleotides long.

11. A method according to claim 1 wherein the capturing probe is between 8 and 15 nucleotides long.

12. A method according to claim 1 wherein more than one capturing LNA-probe is used and the capturing probe consist of different LNA-oligomers directed against different target nucleic acids or against different regions of the same nucleic acid.

13. A method according to claim 12, wherein the different capturing probes are spotted in an array format on the solid surface.

14. A method according to claim 13, wherein the array has at least 10 capturing probes.

15. A method according to claim 13, wherein the array has at least 100 capturing probes.

16. A method according to claim 13, wherein the array has at least 1,000 capturing probes.

17. A method according to claim 13, wherein the array has at least 10,000 capturing probes.

18. A method according to claim 1 wherein the nucleic acids originate from cells, a tissue sample or tissue extract.

19. A method according to claim 18, wherein the cells are of archae, prokaryotic, eukaryotic origin.

20. A method according to claim 18, wherein the sample is derived from blood, serum, plasma, reticulocytes, lymphocytes, urine, bone marrow tissue, cerebrospinal fluid or any product prepared from blood or lymph, muscle biopsy, liver biopsy, kidney biopsy, bladder biopsy, bone biopsy, cartilage biopsy, skin biopsy, pancreas biopsy, a biopsy of the intestinal tract, thymus biopsy, mammae biopsy, uterus biopsy, a testicular biopsy, eye biopsy or a brain biopsy, homogenized in lysis buffer.

21. A method according to claim 1 wherein the capturing probe(s) consist(s) of LNA-oligomer(s) directed against one or more target nucleic acid(s) which is/are specific for a specific species of organisms.

22. A method according to claim 1 wherein the capturing probe(s) consist(s) of LNA-oligomer(s) directed against one or more target nucleic acid(s) which is/are specific for a specific species, sub-species or strain of organisms.

23. A method according to claim 1 wherein the capturing probe(s) consist(s) of LNA-oligomer(s) directed against one or more target nucleic acid(s) which is/are specific for a specific species of micro-organisms.

24. A method according to claim 1 wherein the capturing probe(s) consist(s) of LNA-oligomer(s) directed against one or more target nucleic acid(s) which is/are specific for a specific species, sub-species or strain of micro-organisms.

25. A method according to claim 1 wherein the capturing probe(s) consist(s) of LNA-oligomer(s) directed against one or more target nucleic acid(s) which is/are specific for an infectious agent.

26. A method according to claim 1 wherein the capturing probe(s) consist(s) of LNA-oligomer(s) directed against one or more target nucleic acid(s) which is/are specific for a specific species, sub-species or strain of an infectious agent.

27. A method according to claim 1 wherein the capturing probe(s) consist(s) of LNA-oligomer(s) directed against one or more target nucleic acid(s) which is/are specific for genes coding for proteins involved on an inheritable disease.

28. A method according to claim 1 wherein the capturing probe(s) consist(s) of LNA-oligomer(s) directed against one or more target nucleic acid(s) which is/are specific for genes related to a life style disease.

29. A method according to claim 1 wherein the capturing probe(s) consist(s) of LNA-oligomer(s) directed against one or more target nucleic acid(s) which is/are specific for genes related to cancer.

30. A method according to claim 28, wherein the life style diseases are selected from the group consisting of atherosclerosis and diabetes.

31. A method according to claim 1 wherein the solid surface is selected from the group consisting of glass, carbohydrate polymers and metals.

32. A method according to claim 3 wherein the solid surface is the wall in a microtiter plate.

33. A method according to claim 3 wherein the solid has the form of a bead.

34. A method according to claim 3 wherein the solid surface has the form of a flat plate.

35. A method according to claim 1 wherein the isolation is performed in one step.

36. A method according to any of claims 2 and 3, wherein the ligand is biotin.

37. A method according to claim 1 wherein the target nucleic acids hybridized to the capturing probe are detected by a detection probe.

38. A method according to claim 37, wherein the detection probe is labelled with a label elected from the group consisting of fluorophores, radioactive isotopes, enzymes, ligands and haptenic and antigenic compounds.

39. A method according to claim 38, wherein the fluorophore is selected from the group consisting of fluorescein, rhodamin and Texas Red.

40. A method according to claim 38, wherein the radioactive isotope is selected from the group consisting of ^{32}P, ^{33}P, ^{35}S, ^{3}H, ^{125}I and ^{14}C.

41. A method according to claim 38, wherein the enzyme is selected from the group consisting of horseradish peroxidase, alkaline phosphatase, calf intestine alkaline phosphatase, glucose oxidase and beta-galactosidase.

42. A method according to claim 38, wherein the ligand is selected from the group consisting of biotin, thyroxine and cortisol.

43. A method according to any of claims 37 to 42, wherein the detection probe hybridises to a different region of the immobilised target nucleic acid than the capturing probe.

44. A method according to any of claims 37 to 42, wherein the detecting probe contains at least one LNA-monomer.

45. A method for amplifying a target nucleic acid the nucleotide sequence of which is complementary to the capturing probe, the method comprising the steps of

a) providing a sample containing nucleic acids,

b) treating the sample with a lysing buffer containing a chaotropic agent to lyse cellular material in the sample, dissolve the components and denature the nucleic acids in the sample,

c) contacting the nucleic acids released from the sample with at least one capturing LNA-probe covalently attached to a solid surface, said capturing probe being substantially complementary to the target nucleic acid,

d) separating the solid surface from excess material,

e) combining the captured nucleic acids with an appropriate amount of nucleoside triphosphates and an agent for polymerisation of the nucleoside triphosphates,

f) extending any oligonucleotides which hybridise to the captured nucleic acids to form extension products, wherein the capturing LNA-probes are used as templates,

g) detecting the extension product formed.

46. A method for amplifying a target nucleic acid the nucleotide sequence of which is complementary to the capturing probe, the method comprising the steps of

a) providing a sample containing nucleic acids,

b) treating the sample with a lysing buffer containing a chaotropic agent to lyse cellular material in the sample, dissolve the components and denature the nucleic acids in the sample,

c) contacting the nucleic acids released from the sample with at least one capturing LNA-probe covalently attached to a solid surface, said capturing probe being substantially complementary to the target nucleic acid,

d) separating the solid surface from excess material,

e) combining the captured nucleic acids with an appropriate amount of nucleoside triphosphates and an agent for polymerisation of the nucleoside triphosphates,

f) extending any oligonucleotides which hybridise to the captured nucleic acids to form extension products, wherein the capturing LNA-probes are used as templates,

g) hybridising, in the presence of an appropriate amount of nucleoside triphosphates and an agent for polymerisation of the nucleoside triphosphates, the single stranded nucleic acids from step c) with at least one downstream primer to synthesise further extension products,

h) repeating steps g) through h) a sufficient number of times to result in a detectable amount of extension products,

i) detecting the extension products formed.

47. A method for amplifying a target nucleic acid the nucleotide sequence of which is complementary to the capturing probe, the method comprising the steps of

a) providing a sample containing nucleic acids,

b) treating the sample with a lysing buffer containing a chaotropic agent to lyse cellular material in the sample, dissolve the components and denature the nucleic acids in the sample,

c) contacting the nucleic acids released from the sample with at least one capturing LNA-probe covalently attached to a solid surface, said capturing probe being substantially complementary to the target nucleic acid,

d) separating the solid surface from excess material,

e) combining the captured nucleic acids with an appropriate amount of nucleoside triphosphates, an agent for polymerisation of the nucleoside triphosphates and at least one downstream primer,

f) extending any oligonucleotides which hybridise to the captured nucleic acids to form extension products, wherein said nucleic acids are used as templates,

g) detecting the extension product formed.

48. A method for amplifying a target nucleic acid the nucleotide sequence of which is complementary to the capturing probe, the method comprising the steps of

a) providing a sample containing nucleic acids,

b) treating the sample with a lysing buffer containing a chaotropic agent to lyse cellular material in the sample, dissolve the components and denature the nucleic acids in the sample,

c) contacting the nucleic acids released from the sample with at least one capturing LNA-probe covalently attached to a solid surface, said capturing probe being substantially complementary to the target nucleic acid,

d) separating the solid surface from excess material,

e) combining the captured nucleic acids with an appropriate amount of nucleoside triphosphates, an agent for polymerisation of the nucleoside triphosphates and at least one downstream primer,

f) extending any oligonucleotides which hybridise to the captured nucleic acids to form extension products, wherein said nucleic acids are used as templates,

g) hybridising, in the presence of an appropriate amount of nucleoside triphosphates and an agent for polymerisation of the nucleoside triphosphates, the single stranded nucleic acids from step c) with at least one downstream primer to synthesise further extension products,

h) repeating steps g) through h) a sufficient number of times to result in a detectable amount of extension products,

i) detecting the extension products formed.

49. A kit for isolating a target nucleic acid comprising

a) a lysing buffer containing a chaotropic agent to lyse cellular material in the sample,

b) at least one capturing LNA-probe, said capturing probe being substantially complementary to the target nucleic acid.

50. A method of claim 1 wherein the sample is treated with the chaotropic lysing buffer and the nucleic acids are contacted with the LNA-probe in a single reaction vessel without a wash step between the treating and contacting.

51. A method of claim 50 wherein the lysing buffer comprises a guanidine compound.

52. A method of claim 50 wherein nucleic acid hybridised to the LNA-probe are detected.

53. A method for isolating a target nucleic acid comprising:

a) providing a cellular sample containing nucleic acids;

b) treating the sample with a lysing buffer containing a guanidine compound; and

c) contacting the nucleic acids released from the sample with at least one capturing LNA-probe, the LNA-probe being substantially complementary to the target nucleic acid.

54. A method of claim 53 further comprising detecting nucleic acid hybridised to the LNA-probe.

55. A method of claim 53 wherein the sample is treated with the lysing buffer and the nucleic acids are contacted with the LNA-probe in a single reaction vessel without a wash step between the treating and contacting.

56. A method of claim 54 wherein the sample is treated with the lysing buffer, the nucleic acids are contacted with the LNA-probe, and the nucleic acid hybridized to the LNA-probe are detected in a single reaction vessel without a wash step between the treating, contacting and detecting.

57. A method of claim 53 wherein the guanidine compound is guanidine thiocyanate or guanidine hydrochloride.

58. A method for isolating a target nucleic acid comprising:

a) providing a cellular sample containing nucleic acids;

b) treating the sample with a lysing composition; and

c) contacting the nucleic acids released from the sample with at least one capturing LNA-probe, the LNA-probe containing a total of from 4 to 20 nucleotides and being substantially complementary to the target nucleic acid.

59. A method of claim 58 further comprising detecting nucleic acid hybridised to the LNA-probe.

60. A method of claim 58 wherein the sample is treated with the lysing buffer and the nucleic acids are contacted with the LNA-probe in a single reaction vessel without a wash step between the treating and contacting.

61. A method of claim 58 wherein the sample is treated with the lysing buffer, the nucleic acids are contacted with the LNA-probe, and the nucleic acid hybridized to the LNA-probe are detected in a single reaction vessel without a wash step between the treating, contacting and detecting.

62. A method of claim 58 wherein the lysing composition comprises a guanidine compound.

63. A method of claim 62 wherein the guanidine compound is guanidine thiocyanate or guanidine hydrochloride.

64. A method of claim 58 wherein the LNA probe contains a total of from 8 to 20 nucleotides.

65. A method of claim 58 wherein the LNA probe contains a total of from 8 to 15 nucleotides.

* * * * *

US-PAT-NO: 6303315

DOCUMENT-IDENTIFIER: US 6303315 B1

TITLE: One step sample preparation and detection of nucleic acids in complex biological samples

---------- KWIC ---------

Detailed Description Text - DETX (4):
LNA modified oligos (see table 1-1) carrying a 5' anthraquinone were covalently immobilized to the wells of a microtiter -plate by UV irradiation and used as capture probes in a hybridisation assay with a complementary target DNA oligo. The hybrid was detected by including a 5' biotinylated DNA detection probe in the hybridisation mixture.

Detailed Description Text - DETX (21):
LNA modified oligos (see table 2-1) carrying a 5' anthraquinone were covalently immobilized to the wells of a microtiter -plate by UV irradiation and used as capture probes in a hybridisation assay with a complementary target DNA oligo. The hybrid was detected by including a 5' biotinylated DNA detection probe in the hybridisation mixture.

Detailed Description Text - DETX (44):
LNA modified oligos (see table 3-1) carrying a 5' anthraquinone were covalently immobilized to the wells of a microtiter -plate by UV irradiation and used as capture probes in a hybridisation assay with a complementary target DNA oligo. The hybrid was detected by including a 5' biotinylated DNA detection probe in the hybridisation mixture.

Detailed Description Text - DETX (66):

LNA modified oligos (see table 2-1) carrying a 5' anthraquinone were covalently immobilized to the wells of a microtiter -plate by UV irradiation and used as capture probes in a hybridisation assay with a complementary target DNA oligo. The hybrid was detected by including a 5' biotinylated DNA detection probe in the hybridisation mixture.

Detailed Description Text - DETX (152):

LNA modified oligos (see table 7-2) carrying a 5' anthraquinone were covalently immobilized to the wells of a microtiter -plate by UV irradiation and used as capture probes in a hybridisation assay with a complementary target DNA oligo. The hybrid was detected by including a 5' biotinylated DNA detection probe in the post-hybridisation mixture.

US005563036A

United States Patent [19]

Peterson et al.

[11] **Patent Number: 5,563,036**

[45] **Date of Patent: Oct. 8, 1996**

[54] **TRANSCRIPTION FACTOR-DNA BINDING ASSAY**

[75] Inventors: **Michael G. Peterson; Vijay R. Baichwal; Berta Strulovici**, all of So. San Francisco, Calif.

[73] Assignee: **Tularik, Inc.**, South San Francisco, Calif.

[21] Appl. No.: **235,503**

[22] Filed: **Apr. 29, 1994**

[51] **Int. Cl.**[6] **C12Q 1/68**

[52] **U.S. Cl.** **435/6**

[58] **Field of Search** 435/6

[56] **References Cited**

U.S. PATENT DOCUMENTS

4,789,628	12/1988	Nayak	435/7.94
4,816,730	3/1989	Wilhelm, Jr. et al.	318/568.22
5,200,051	4/1993	Cozzette et al.	204/403
5,453,362	9/1995	Lamarco et al.	435/69.1

FOREIGN PATENT DOCUMENTS

46637/89 12/1989 WIPO .

OTHER PUBLICATIONS

Kemp et al., "Simplified Colorimetric Analysis of Polymerase Chain Reactions: Detection of HIV Sequences in AIDS Patients", *Gene* 94:223–228 (1990).

Kemp et al., "Colorimetric Detection of Specific DNA Segments Amplified by Polymerase Chain Reactions", *Proc. Natl. Acad. Sci. USA* 86:2423–2427 (1989).

Jost et al., Nucleic Acids Research, vol. 19(10): p. 2788, (1991).

Gambari et al., Proceedings of the American Association for Cancer Research, vol. 32: p. 333, (1991).

Peterson et al., Trends in Biotechnology, vol. 11(1): pp. 11–18, (Jan., 1993).

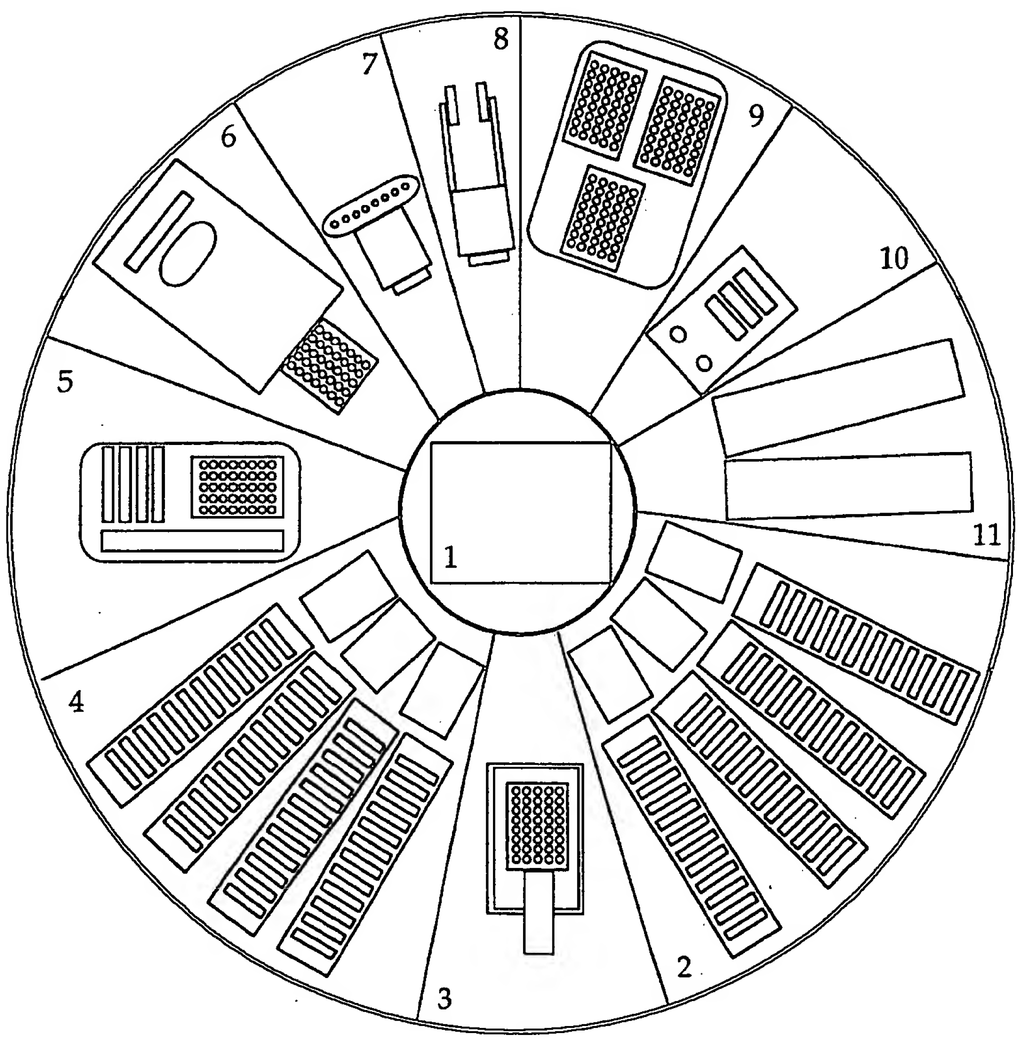
Primary Examiner—George C. Elliott
Assistant Examiner—Terry A. McKelvey
Attorney, Agent, or Firm—Flehr, Hohbach, Test, Albritton & Herbert

[57] **ABSTRACT**

Pharmacological agents useful in the diagnosis or treatment of disease associated with the expression of a gene are identified in high throughput drug screening assays. The methods involve combining a labeled transcription factor, a nucleic acid coupled to a ligand, a candidate pharmacological agent and a receptor immobilized on a solid substrate, such as a microtiter plate, filter, or bead. The nucleic acid has at least that portion of a nucleotide sequence naturally involved in the regulation of the transcription of the gene which is necessary for sequence-specific interaction with the transcription factor. The resultant combination is incubated under conditions whereby the receptor is bound to the ligand and, but for the presence of said candidate pharmacological agent, the transcription factor is sequence-specifically bound to the nucleic acid. Unbound transcription factor is then removed or washed from the solid substrate and labelled, sequence-specifically bound transcription factor is detected. Incubates which include candidate agents which alter transcription factor binding deviate from control incubates in terms of label signal—typically, binding is disrupted and the signal is diminished. In a preferred embodiment, the entire process is performed by a computer-controllable electromechanical robot with an axial rotatable arm.

14 Claims, 1 Drawing Sheet

FIGURE 1



TRANSCRIPTION FACTOR-DNA BINDING ASSAY

INTRODUCTION

1. Field of the Invention

The field of this invention is assays for screening for drugs which interfere with sequence-specific protein-DNA binding.

2. Background

Half the U.S. population is infected with Herpes Simplex Virus (HSV) type 1. A quarter of the U.S. population is infected with HSV type 2. Over a million Americans are infected with HIV. About 1% of all newborns in the U.S. have congenital cytomegalovirus infection. The numbers are staggering, yet effective therapeutics are unavailable for these and most other viral infections. A similar deficiency is found in treatments of heart disease, cancer and many other of significant threats to modern human health.

Gene-specific transcription factors provide a promising class of targets for novel therapeutics directed to these and other human diseases for the following reasons. One, transcription factors offer substantial diversity. Over 300 gene-specific transcription factors have been described, and the human genome may encode as many as 3000. Hence, they provide as plentiful a target source as cell-surface receptors. Two, transcription factors offer substantial specificity. Each and every factor offers unique molecular surfaces to target. Three, transcription factors are known to be involved in human disease. For example, many tumors are associated with the activation of a specific oncogene. A third of known proto-oncogenes and three fourths of all anti-oncogenes are transcription factors.

A number of extremely effective presently marketed drugs act, at least indirectly, by modulating gene transcription. For instance, in many cases of heart disease, the LDL receptor is pathogenically down-regulated at the level of transcription by intracellular sterol levels. The drug compactin, an inhibitor of HMC CoA reductase, functions by up-regulating transcription of the LDL receptor gene which leads to clearance of cholesterol from the blood stream.

Identifying and developing new pharmaceuticals is a multibillion dollar industry in the U.S. alone. Urgently needed are efficient methods of identifying pharmacological agents or drugs which are active at the level of gene transcription. If amenable to automated, cost-effective, high throughput drug screening, such methods would have immediate application in a broad range of domestic and international pharmaceutical and biotechnology drug development programs.

Relevant Literature

Kemp, D. J., Foote, S. J., Peterson, M. G., Samaras, N., and Smith, D. et at. (1989) Amplified DNA Assay. PCT International Application Number 46637/89 (Filed 8 Dec. 1989).

Kemp, D. J., et at. (1990) Simplified colorimetric analysis of polymerase chain reactions: detection of HIV sequences in AIDS patients. Gene 94, 223–228.

Kemp, D. J. et at. (1989) Colorimetric detection of specific DNA segments amplified by polymerase chain reactions. PNAS USA 86, 2423–2427.

SUMMARY OF THE INVENTION

The invention provides methods for identifying pharmacological agents useful in the diagnosis or treatment of disease associated with the expression of a gene.

In general, the methods involve combining a labeled protein, a nucleic acid, a candidate pharmacological agent and a receptor immobilized on a solid substrate, such as a microtiter plate. The labelled protein includes at least a portion of a natural transcription factor involved in the regulation of the gene's expression. The nucleic acid has at least that portion of a nucleotide sequence naturally involved in the regulation of the transcription of the gene which is necessary for sequence-specific interaction, direct or indirect, with the transcription factor. The nucleic acid is conjugated to a ligand capable of specificity binding the immobilized receptor. The resultant mixture is incubated under conditions whereby the receptor is bound to the ligand and, but for the presence of said candidate pharmacological agent, the transcription factor is sequence-specifically bound to the nucleic acid. Unbound transcription factor is then removed or washed from the solid substrate and labelled, sequence-specifically bound transcription factor is detected. Binding reactions, "incubates", which include candidate agents which alter transcription factor binding deviate from control incubates in terms of label retained on the substrate—typically, binding is disrupted and the signal is diminished. In this way, pharmacological agents which modulate transcription factor-gene interactions are identified.

A wide variety of alternative embodiments of the general method are disclosed. These include a variety of labels, ligands, receptors, genes, transcription factors, auxiliary factors, etc. In a preferred embodiment, the transcription factor is vital or eukaryotic, the label is a radioactive atom, the receptor is avidin and the ligand is biotin. Much of the method is amenable to performance by electromechanical robot. In a preferred embodiment, the method is performed by a computer-controllable electromechanical robot with an axial rotatable arm. In addition, the invention provides kits for drug screening based on the disclosed nucleic acid binding methods.

BRIEF DESCRIPTION OF THE FIGURE

FIG. 1: Schematic of robotic station design.

DETAILED DESCRIPTION OF THE INVENTION

The invention provides efficient methods of identifying pharmacological agents or drugs which are active at the level of gene transcription. The methods are amenable to automated, cost-effective high throughput drug screening and have immediate application in a broad range of domestic and international pharmaceutical and biotechnology drug development programs.

Target diseases are limited only in that disease or disease progression be subject to inhibition by alteration of the specific interaction of a transcription factor and a gene or gene regulatory region. As such, target diseases include viral, bacterial and fungal infections, metabolic disease, genetic disease, cell growth and regulatory disfunction, such as neoplasia, inflammation, hypersensitivity, etc. The target diseases may be afflictions of plants, especially agricultural crops, or animals, especially livestock, house animals and humans.

Transcription factors arc capable of sequence-specific interaction with a portion of a genc or gene regulatory region. The interaction may be direct sequence-specific binding where the transcription factor directly contacts the nucleic acid or indirect sequence-specific binding mediated or facilitated by other auxiliary proteins where the transcription factor is tethered to the nucleic acid by a direct nucleic acid binding protein. In addition, some transcription factor demonstrate induced or synergistic binding. A broad range of transcription factor-nucleic acid complexes provide useful targets. The gene and/or transcription factor may be derived from a host or from an infectious or parasitic organism. As examples, a host may be immunomodulated (e.g. by controlling inflammation or hypersensitivity) by modulating the DNA binding of a transcription factor involved in immune cell activation; or vital, bacterial, or other microbial disease progression may be inhibited by disrupting the DNA binding of a host, vital or other microbial transcription factor involved in vital or other microbial gene transcription.

Applicable host and vital or microbial transcription factors and corresponding oligonucleotide targets are found in sources such as the regularly updated Transcription Factor Databasc of the National Center for Biotechnology Information at the National Library for Medicine and Faisst and Meyer (1991) Nucleic Acids Research 20, 3–26. Preferred pain are listed in Table 1 below.

Factor[1]	Binding Sequence[3]
AAF	TTTCATATTACTCT (SEQ ID NO:1)
AhR	TGCGTGAGAAGA (SEQ ID NO:2)
Apl	TGASTMA
AP2	CCCMCNSSS
AP3	TGTGGWWW
AP4	YCAGCTGYGG (SEQ ID NO:3)
AR	AGAACANNNTGTTCT (SEQ ID NO:4)
ARP-1	TGANCCCTTGACCCCT (SEQ ID NO:5)
ATF	TGACGYMR
BGPl	GGGGGGGGGGGGGGG (SEQ ID NO:6)
BSAP	GACGCANYGRWNNNMG (SEQ ID NO:7)
CBF	ACACCCAAATATGGCGAC (SEQ ID NO:8)
C/EBP	GTGGWWWG
CFl	ANATGG
COUP	GTGTCAAAGGTCA (SEQ ID NO:9)
CPl	YNNNNNNRRCCAATCANYK (SEQ ID NO:10)
CP2	YAGYNNNRRCCAATCNNNR (SEQ ID NO:11)
CTCF	CCCTC
DBP	TGATTTTGT
E2A	RCAGNTG
E2B	TGCAAYAY
E2F	TTTTSSCGS
E4F	TGACGTAAC
EGR-1	CGCCCSCGC
EGR-2	CCGCCCCCGC (SEQ ID NO:12)
ER	AGGTCANNNTGACCT (SEQ ID NO:13)
v-ErbA	GTGTCAAAGGTCA (SEQ ID NO:14)
ETF	CAGCCCCCGCGCAGC (SEQ ID NO:15)
Ets-1	SMGGAWGY
F-ACTl	TGGCGA
GATA-1	WGATAR
GATA-2	WGATAR
GATA-3	WGATAR
GCF	SCGSSSC
GHF-1	WTATYCAT
GHF-5	WTATYCAT
GHF-7	WTATYCAT
GR	AGAACANNNTGTTCT (SEQ ID NO:16)

-continued

Factor[1]	Binding Sequence[3]
H1TF2	GCACCAATCACAGCGCGC (SEQ ID NO:17)
H2RIIBP	TCAGGTCACAGTGACCTGA (SEQ ID NO:18)
H2TFl	TGGGGATTCCCCA (SEQ ID NO:19)
H-APF-1	CTGGRAA
HNF-1	GTTAATNATTAAC (SEQ ID NO:20)
vHNF-1	GTTAATNATTAAC (SEQ ID NO:20)
HNF-3A	TATTGAYTTWG (SEQ ID NO:21)
HNF-3B	TATTGAYTTWG (SEQ ID NO:21)
HNF-3C	TATTGAYTTWG (SEQ ID NO:21)
HNF-4	KGCWARGKYCAY (SEQ ID NO:22)
HSF	NGAANNGAANNGAAN (SEQ ID NO:23)
IAF	GCCATCTGCT (SEQ ID NO:24)
IREBF-1	CGGGAAATGGAAACTG (SEQ ID NO:25)
IRBP	AGTGCACT
ISGF1	CTTTCAGTTT (SEQ ID NO:26)
ISGF2	CTTTCTCTTT (SEQ ID NO:27)
ISGF3	GCTTCAGTTT (SEQ ID NO:28)
KBF-1	TGGGGATTCCCCA (SEQ ID NO:29)
Kerl	GCCTGCAGGC (SEQ ID NO:30)
LFB3	GTTAATNATTAAC (SEQ ID NO:31)
LIT-1	GCGCCCTTTGGACCT (SEQ ID NO:32)
LyF-1	PPTGGGAGR
MBF-1	YTAAAAATAAYYY (SEQ ID NO:33)
MBF-I	TGCRCRC
MBP-1	TGGGGATTCCCCA (SEQ ID NO:34)
MCBF	CATTCCT
MEF-2	YTAWAAATAR (SEQ ID NO:35)
MEP-1	TGCRCNC
MR	AGAACANNNNTGTTCT (SEQ ID NO:36)
Myb	YAACKG
Myc	CACGTG
	TCTCTTA
MyoD	CAACTGAC
NF1	YGGMNNNNNGCCAA (SEQ ID NO:37)
NF-AT	GGAGGAAAAACTGTTTCAT (SEQ ID NO:38)
NF-E2	TGACTCAG
NF-D	GATGGCGG
NF-GMa	GRGRGTTKCAY (SEQ ID NO:39)
NF-GMb	TCAGRTA
NF-IL6	TKNNGNAAK
NFκB	GGGAMTNYCC (SEQ ID NO:40)
NF-W1	GTTGCATC
NF-W2	GTTGCATC
NGF1-B	AGGTCATGACCT (SEQ ID NO:41)
Oct-1	ATGCAAAT
Oct-2	ATGCAAAT
Oct-4	ATGCWAAT
Oct-6	ATGCAAAT
Pax-1	CACCGTTCCGCTCTAGATATCTC (SEQ ID NO:42)
PCF	AGAAAGGGAAAGGA (SEQ ID NO:43)
PEA3	AGGAAR
PPAR	AGGTCA
PR	AGAACANNNTGTTCT (SEQ ID NO:44)
PRDI-BF1	AAGTGAAAGT (SEQ ID NO:45)
PTF1	ATGGGANCTCAGCTGTGC (SEQ ID NO:46)
Pu.I	AGAGGAACT
PuF	GGGTGGG
RAR	AGGTCATGACCT (SEQ ID NO:47)
RFX	CCCCTAGCAACAGATG (SEQ ID NO:48)
RVF	AAGATAAAACC (SEQ ID NO:49)
SIF	CCCGTM
Sp1	KRGGCTRRK
SRF	GGATGTCCATATTAGGACATCT (SEQ ID NO:50)
TBP	TATAAA
TCF-1	MAMAG
TCF-2α	SAGGAAGY
TEF-1	AAGYATGCA
TEF-2	GGGTGTGG

-continued

Factor[1]	Binding Sequence[3]
TGT3	AAGTGTTTGC (SEQ ID NO:51)
TIN-1	AGGAAGTTCC (SEQ ID NO:52)
WT-ZFP	CGCCCCCGC
XF1/2	TCTTCTCACGCAACT (SEQ ID NO:53)
XPF-1	CACCTGNNNNTTTCCC (SEQ ID NO:54)
YB-1	ATTTTTCTGATTGGCCAAAG (SEQ ID NO:55)
Epstein-Barr Virus EBNA (B958 strain)	GGT TAG CAT ATG CTA ACC A (SEQ ID NO:56)
Epstein-Barr Virus BZLF (B958 strain)	T TAG CAA TG
Human CBF-1	CGTGGGAA (EpsteinBarr Virus cis-element)
Human Papilloma Virus E2 (strain 6)	A CCG AAA ACG GTG T (SEQ ID NO:57)
Herpes Simplex Virus Type 1 VP16	ATG CTA ATG ATA (SEQ ID NO:58)
HIV TAT	GGG TCT CTC TGG TTA GAC CAG ATC TGA GCC TGG GAG CTC TCT GGC TAA CTA GGG AAC CCA (SEQ ID NO:59) (TAR RNA SEQUENCE)

The disclosed methods and kits involve reconstituting, in vitro, sequence-specific transcription factor-nucleic acid interactions, and challenging the reconstitution with candidate therapeutics. Preferred applications of the method include gene transcriptional regulation where at least one transcription factor and corresponding gene or gene regulatory region have been molecularly cloned. The methods involve forming a mixture of a labelled protein comprising at least a potion of a transcription factor, a nucleic acid conjugated to a ligand, a candidate pharmacological agent and a receptor immobilized on a solid substrate.

The labelled protein comprises at least a portion of a transcription factor and a label, the potion being sufficient to permit sequence-specific binding, direct or indirect, of the labelled protein to the nucleic acid conjugate. The portion is usually at least about 20, more usually at least about 40, most usually at least about 80 amino acids in length and includes residues sufficient to provide the protein with sequence-specificity similar to that of the native transcription factor. Frequently, the labelled protein will include the entire transcription factor. The labelled protein is typically capable of binding the nucleic acid conjugate with an equilibrium constant at least about $10^4 M^{-1}$, preferably at least about $10^6 M^{-1}$, more preferably at least about $10^8 M^{-1}$ and not less than six, preferably not less than four, more preferably not less than two orders of magnitude less than the binding equilibrium constant of the native transcription factor under similar conditions.

Preferred transcription factor portions capable of imparting the requisite binding specificity and affinity are readily identified by those skilled in the art. A wide variety of molecular and biochemical methods are available for generating preferred portions, see e.g. Molecular Cloning, A Laboratory Manual (2nd Ed., Sambrook, Fritsch and Maniatis, Cold Spring Harbor), Current Protocols in Molecular Biology (Eds. Aufubel, Brent, Kingston, More, Feidman, Smith and Stuhl, Greene Publ. Assoc., Wiley-Interscience, NY, N.Y., 1992) or that are otherwise known in the art. For example, deletion routants are screened for sequence-specific binding directly using a label or through gel shift analysis.

The labelled protein also comprises a label which is used to detect labelled protein-nucleic acid complexes. A wide variety of labels may be employed—essentially any label that provides for detection of the labelled protein when complexed to the nucleic acid conjugate. The label may provide for direct detection as radioactivity, luminescence, optical or electron density, etc. or indirect detection such as an epitope tag, an enzyme, etc. The label may be appended to the protein e.g. a phosphate group comprising a radioactive isotope of phosphorous, or incorporated into the protein structure, e.g. a methionine residue comprising a radioactive isotope of sulfur.

The protein may also comprise additional components depending upon the assay reagents and conditions. For example, it may be desirable that the protein be a fusion product of the transcription factor portion and another polypeptide, e.g. a polypeptide that is capable of providing or enhancing sequence-specific nucleic acid binding or stability under assay conditions.

The nucleic acid conjugate comprises a nucleic acid coupled to a ligand. The nucleic acid is usually linear and double-stranded DNA or RNA, particularly in the case of retroviral transcription factor binding sites, though circular plasmids or other nucleic acids or structural analogs may be substituted so long as transcription factor sequence-specific binding is retained. In some applications, supercoiled DNA provides optimal sequence-specific binding and is preferred. The nucleic acid may be of any length amenable to the assay conditions and requirements. Typically the nucleic acid is between 8 bp and 5 kb, preferably between about 12 bp and 1 kb, more preferably between about 18 bp and 250 bp, most preferably between about 27 and 50 bp.

The nucleic acid has a sequence at least a portion of which is common to the gene or gene regulatory region to which the native transcription factor normally binds. The portion may be continuous or segmented and shares sufficient sequence and sequence similarity with the gene or gene regulatory region to provide sequence-specific binding of the labelled protein. Typically, this binding site portion of the nucleic acid constitutes at least about 4, preferably at least about 6, more preferably at least about 8 nucleotides. Additional nucleotides may used to provide structure which enhances or decreased binding or stability, etc. For example, combinatorial DNA binding can be effected by including two or more DNA binding sites for different or the same transcription factor on the oligonucleotide. This allows for the study of cooperative or synergistic DNA binding of two or more factors, eg. HPV E1 and E2 bind cooperatively to DNA by virtue of their protein-protein interaction. In addition, the nucleic acid can comprise a cassette into which transcription factor binding sites are conveniently spliced for use in the subject assays. An exemplary cassette showing how new DNA binding sites can be exchanged in a simple fashion within a common 30-mer sequence appears in Table 2 below.

TABLE 2

EBV EBNA-1 site	GGA TCT GGT TAG CAT ATG CTA ACC AGG ATC (SEQ ID NO:60)
HPV E2 substituted	GGA TCT GGT ACC GAA AAC GGT ACC AGG ATC (SEQ ID NO:61)

TABLE 2-continued

EBV BZLF-1 substituted	GGA TCT GGT TAG TTA GCA ATG ACC AGG ATC (SEQ ID NO:62)
Nf-kB and homologs	GGA TCT GGT TAG GGG ATT TCC ACC AGG ATC (SEQ ID NO:63)
HSV VP16 cis-element	GGA TCT GGT TAT GCT AAT GAT ATC AGG ATC (SEQ ID NO:64)

The ligand of the nucleic acid conjugate is capable of specifically binding the immobilized receptor. The ligand-receptor binding is specific enough to provide a maximized and at least measurable signal to noise ratio (receptor mediated vs. non-specific retention of the label on the substrate). The nucleic acid conjugate is typically capable of binding the receptor with an affinity of at least about 10^5M^{-1}, preferably at least about 10^6M^{-1}, more preferably at least about 10^8M^{-1}. In a preferred embodiment, a plurality of ligands are capable of binding each receptor. Exemplary ligand-receptor pairs include biotin and avidin, antigen and antibody, sugar and lectin, ion and chelator, etc.

The receptor is immobilized on a solid substrate which may be any solid from which the unbound labelled protein may be conveniently separated. The solid substrate may be made of a wide variety of materials and in a wide variety of shapes, e.g. microfiter plate, microbead, dipstick, resin particle, etc. The substrate is chosen to maximize signal to noise ratios, primarily to minimize background binding, for ease of washing and cost. For example, beads with iron cores may be readily isolated (washed) using magnets.

The mixture aim comprises a candidate pharmacological agent. Candidate agents encompass numerous chemical classes, though typically they are organic compounds; preferably small organic compounds. Small organic compounds have a molecular weight of more than 50 yet less than about 2,500, preferably less than about 1000, more preferably, less than about 500. Candidate agents comprise functional chemical groups necessary for structural interactions with proteins and/or DNA, and typically include at least an amine, carbonyl, hydroxyl or carboxyl group, preferably at least two of said functional chemical groups, more preferably at least three. The candidate agents often comprise cyclical carbon or heterocyclic structures and/or aromatic or polyaromatic structures substituted with one or more of the forementioned functional groups. Candidate agents are also found among biomolecules including peptides, saccharides, fatty acids, steroids, purines, pyrimidines, derivatives, structural analogs or combinations thereof, and the like.

Candidate agents are obtained from a wide variety of sources including libraries of synthetic or natural compounds. For example, numerous means are available for random and directed synthesis of a wide variety of organic compounds and biomolecules, including expression of randomized oligonucleotides. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural and synthetically produced libraries and compounds are readily modified through conventional chemical, physical, and biochemical means. In addition, known pharmacological agents may be subject to directed or random chemical modifications, such as acylation, alkylation, esterification, amidification, etc., to produce structural analogs.

In addition to the labelled protein, nucleic acid conjugate, candidate agent and immobilized receptor, the mixture usually includes additional reagents, such as salts, buffers, etc. to facilitate optimal receptor-ligand and protein-nucleic acid binding. Auxiliary proteins or portions thereof may also be included to mediate, facilitate or otherwise enhance sequence-specific protein-nucleic acid binding. For example, sequence-specific binding of a number of viral transcription factors is enhanced when complexed with one or more cellular proteins, e.g. Octl and HCF in the case of HSV's VP16. Other exemplary auxiliary proteins include CBF1, for EBNA-2 binding, ATF-2 or AP-1 for Adenovirus E1A binding, etc.

A variety of other reagents may also be included in the mixture. These include reagents like detergents which may be used to reduce non-specific or background protein-substrate, nucleic acid-substrate, protein-protein and protein-DNA interactions, etc. Also, reagents that otherwise improve the efficiency of the assay, such as protease inhibitors, nuclease inhibitors, antimicrobial agents, etc. may be used.

The mixture is incubated under conditions whereby the receptor is bound to the ligand and, but for the presence of said candidate pharmacological agent, the labelled protein is sequence-specifically bound to the nucleic acid. The mixture components can be added in any order that provides for the requisite bindings. For example, the nucleic acid conjugate may be added first and prebound to the substrate through ligand-receptor binding before the labelled protein is added. Alternatively, the nucleic acid conjugate and labelled protein can be preincubated and complexed and then added to the substrate for attachment, or the various mixture components and reagents can be added to the substrate simultaneously. Adding the protein and nucleic acid components together may be thermodynamically advantageous in that in some nucleic acid-protein complexes, initial binding may be favored by a soluble, unrestrained nucleic acid molecule.

Incubations may be performed at any temperature which facilitates optimal binding, typically between 4° and 40° C., more commonly between 15° and 40° C. Incubation periods are likewise selected for optimal binding but also minimized to facilitate rapid, high-throughput screening. Typically, protein-nucleic acid and receptor-ligand pairs are coincubated between 0.1 and 10 hours, preferably less than 5 hours, more preferably less than 2 hours each; of course, the incubations may and preferably do run simultaneously.

After receptor-ligand and protein-nucleic acid binding have occurred, a fraction comprising labelled protein which is not sequence-specifically bound is separated from the solid substrate. This step may be accomplished in a variety of ways including removing a bead or dipstick from a reservoir, emptying or diluting reservoir such as a microtiter plate well, rinsing a bead, particle, chromatographic column or filter with a wash solution or solvent. Typically, the separation step will include an extended rinse or wash or a plurality of rinses or washes. For example, where the solid substrate is a microtiter plate, the wells may be washed several times with a washing solution, which typically includes those components of the incubation mixture that do not participate in specific binding such as salts, buffer, detergent, nonspecific protein, etc.

After separating the unbound fraction from the solid substrate, the presence of bound nucleic acid-protein complex is detected via the labeled protein. A variety of methods may be used to detect the label depending on the nature of

the label and other assay components. For example, the label may be detected bound to the solid substrate or a portion of the bound complex containing the label may be separated from the solid substrate, and thereafter the label detected. Labels may be directly detected through optical or electron density, radiative emissions, nonradiative energy transfers, etc. or indirectly detected with antibody conjugates, etc. For example, in the case of radioactive labels, emissions may be detected directly, e.g. with particle counters or indirectly, e.g. with scintillation cocktails and counters.

Candidate agents shown to modulate transcription complex formation provide valuable reagents to the pharmaceutical and agricultural industries for cellular, plant, field crop, animal and human trials.

The methods are particularly suited to automated high throughput drug screening. In a preferred embodiment, the individual sample incubation volumes are less than about 500 ul, preferably less than about 250 ul, more preferably less than about 100 ul. Such small sample volumes minimize the use of often scarce candidate agent, expensive transcription complex components, and hazardous radioactive waste. Furthermore, the methods provide for automation, especially computerized automation. Accordingly, the method steps are preferably performed by a computer-controlled electromechanical robot. While individual steps may be separately automated, a preferred embodiment provides a single computer-controlled multifunction robot with a single arm axially rotating to and from a plurality of work stations performing the mixture forming, incubating and separating steps. The computer is loaded with software which provides the instructions which direct the arm and work station operations and provides input (e.g. keyboard and/or mouse) and display (e.g. monitor) means for operator interfacing.

In a particular embodiment, the robotic station comprises a robotic arm **1** with axially-positioned work stations including a working source plate station **2**, a working pipette tip station **3**, a working assay plate station **4**, a liquid dispensing station **5**, a wash station **6**, an eight channel pipettor station **7**, a grip hand station **8**, a shaker station **9**, a cooling station **10** and a pipet tip storage station **11**. The arm retrieves and transfers a microtiler plate to a liquid dispensing station where measured aliquots of each an incubation buffer and a solution comprising one or more candidate agents are deposited into each designated well. The arm then retrieves and transfers to and deposits in designated wells a measured aliquot of a solution comprising a labeled transcription factor protein. After a first incubation period, the liquid dispensing station deposits in each designated well a measured aliquot of a biotinylated nucleic acid solution. The first and/or following second incubation may optionally occur after the arm transfers the plate to a shaker station. After a second incubation period, the arm transfers the microtiter plate to a wash station where the unbound contents of each well is aspirated and then the well repeatedly filled with a wash buffer and aspirated. Where the bound label is radioactive phosphorous, the arm retrieves and transfers the plate to the liquid dispensing station where a measured aliquot of a scintillation cocktail is deposited in each designated well. Thereafter, the mount of label retained in each designated well is quantified.

In more preferred embodiments, the liquid dispensing station and arm are capable of depositing aliquots in at least eight wells simultaneously and the wash station is capable of filling and aspirating ninety-six wells simultaneously. Preferred robots are capable of processing at least 640 and preferably at least about 1,280 candidate agents every 24 hours, e.g. in microtiter plates.

The following examples are offered by way of illustration and not by way of limitation.

EXAMPLES

GENERIC PROTOCOL FOR TRANSCRIPTION FACTOR-DNA BINDING ASSAY

1. Reagents:

Neutralite Avidin: 20% μg/ml in PBS.

Blocking buffer: 5% BSA, 0.5% Tween 20 in PBS; 1 hr, RT.

Assay Buffer: 100 mM KCl, 20 mM HEPES pH 7.6, 0.25 mM EDTA, 1% glycerol, 0.5% NP-40, 50 mM BME, 1 mg/ml BSA, cocktail of protease inhibitors.

^{33}P Full-Length Transcription Factor 10× stock: 1–5×10^{-8} "cold" protein comprising unlabeled protein comprising transcription factor portion supplemented with 100,000–500,000 cpm of labeled protein (Beckman counter). Placed in the 4° C. microfridge during screening.

Protease inhibitor cocktail (1000×): 10 mg Trypsin Inhibitor (BMB #109894), 10 mg Aprotinin (BMB #236624), 25 mg Benzamidine (Sigma #B-6506), 25 mg Leupeptin (BMB #1017128), 10 mg APMSF (BMB #917575), and 2 mM $NaVo_3$ (Sigma #S-6508) in 10 ml of PBS.

Oligonucleotide stock: (specific biotinylated). Biotinylated oligo at 1–100 pmole/μl, including transcription factor binding site: (BIOTIN)-oligo: e.g. derived from Table 1. anti-sense: derived as reverse complement of target oligo above.

2. Preparation of assay plates:

Coat with 100 μl of stock N-Avidin per well overnight at 4° C.

Wash 2× with 200 μl PBS.

Block with 150 μl of blocking buffer.

Wash 2× with 200 μl PBS.

3. Assay:

Add 40 μl assay buffer/well.

Add 10 μl compound or extract.

Add 10 μl ^{33}P-labelled protein (10,000–50,000 cpm/well; 10^{-10}–10^{-8}M final concentration).

Shake at RT for 15 min.

Incubate additional 45 min. at 25 C.

Add 40 μl oligo mixture (1.0 pmoles/40 ul in assay buffer)

Incubate 1 hr at 25 C.

Stop the reaction by washing 4× with 200 μl PBS.

Add 150 μl scintillation cocktail.

Count in Topcount.

PROTOCOL FOR EPSTEIN BARR VIRUS EBNA-1 BINDING ASSAY

1. Reagents:

Neutralite Avidin: 20 μg/ml in PBS.

Blocking buffer: 5% BSA, 0.5% Tween 20 in PBS; 1 hr, RT.

Assay Buffer: 100 mM KCl, 20 mM HEPES pH 7.6, 0.25 mM EDTA, 1% glycerol, 0.5% NP-40, 50 mM BME, 1 mg/ml BSA, cocktail of protease inhibitors.

^{33}P EBNA 10× stock: 3×10^{-8} "cold" EBNA (M.W. ~40,000 for dimer) supplemented with 200,000–250,000 cpm of labeled EBNA-1 (Beckman counter). This is to be placed in the 4° C. microfridge during screening.

Protease inhibitor cocktail (1000×): 10 mg Trypsin Inhibitor (BMB #109894), 10 mg Aprotinin (BMB #236624), 25 mg Benzamidine (Sigma #B-6506), 25 mg Leupeptin (BMB #1017128), 10 mg APMSF (BMB #917575), and 2 mM $NaVo_3$ (Sigma #S-6508) in 10 ml of PBS.

Oligonucleotide stock: (specific biotinylated). Biotinylated oligo at 17 pmole/μl, EBNA site TO889/832: (BIOTIND-GGA TCT GGT TAG CAT ATG CTA ACC AGG ATC (SEQ ID NO: 60) anti-sense-GAT CIT GGT TAG CAT ATG CTA ACC AGA TCC (SEQ ID NO: 65)

2. Preparation of assay plates:

Coat with 100 μl of stock N-Avidin per well overnight at 4° C.

Wash 2× with 200 μl PBS.

Block with 150 μl of blocking buffer.

Wash 2× with 200 μl PBS.

3. Assay:

Add 40 μl assay buffer/well.

Add 10 μl compound or extract.

Add 10 μl ^{33}P-EBNA-1 (20,000–25,000 cpm/0.3 pmoles/well=3×10^{-9}M final concentration).

Shake at RT for 15 min.

Incubate additional 45 min. at RT.

Add 40 μl oligo mixture (1.0 pmoles/40 ul in assay buffer).

Incubate 1 hr at RT.

Stop the reaction by washing 4× with 200 μl PBS.

Add 150 μl scintillation cocktail.

Count in Topcount.

PROTOCOL FOR EPSTEIN BARR VIRUS BZLF-1 BINDING ASSAY

1. Reagents:

Neutralite Avidin: 20 μg/ml in PBS.

Blocking buffer: 5% BSA, 0.5% Tween 20 in PBS; 1 hr, RT.

Assay Buffer: 100 mM KCl, 20 mM HEPES pH 7.6, 0.25 mM EDTA, 1% glycerol, 0.5% NP-40, 50 mM BME, 1 mg/ml BSA, cocktail of protease inhibitors.

^{33}P Full-Length BZLF 10× stock: 1×10^{-8} "cold" BZLF supplemented with 180,000–220,000 cpm of labeled BZLF (Beckman counter), resulting in an approx. specific activity of 180,000–220,000 cpm/1 pmole (M.W. ~54,000 for dimer). The protein stock solution contains 70% Et-OH, 30% assay buffer without BSA, and 50 mM BME (final concentration). The protein is to be placed in the 4° C. microfridge during screening.

Protease inhibitor cocktail (1000×): 10 mg Trypsin Inhibitor (BMB #109894), 10 mg Aprotinin (BMB #236624), 25 mg Benzamidine (Sigma #B-6506), 25 mg Leupeptin (BMB #1017128), 10 mg APMSF (BMB #917575), and 2 mM $NaVo_3$ (Sigma #S-6508) in 10 ml of PBS.

Oligonucleotide stock: (specific biotinylated). Biotinylated oligo at 22 pmole/μl, BZLF site TO855/854: sense-(BIOTIN) TTAT CTA CAT TAG CAA TGC CTT AGC AAT GTG CAT A (SEQ ID NO: 66) anti-sense-TAT GCA CAT TGC TAA GGC ATE GCT AAT GTA GAT A (SEQ ID NO: 67)

2. Preparation of assay plates:

Coat with 100 μl of stock N-Avidin per well overnight at 4° C.

Wash 2× with 200 μl PBS.

Block with 150 μl of blocking buffer.

Wash 2× with 200 μl PBS.

3. Assay:

Add 40 μl assay buffer/well.

Add 10 μl compound or extract.

Add 10 μl ^{33}P-BZLF (18,000–22,000 cpm/0.1 pmoles/well=1×10^{-9}M final concentration).

Shake at RT for 15 min.

Incubate additional 45 min. at RT.

Add 40 μl oligo mixture (1.0 pmoles/40 ul in assay buffer)

Incubate 1 hr at RT.

Stop the reaction by washing 4× with 200 μl PBS.

Add 150 μl scintillation cocktail.

Count in Topcount.

PROTOCOL FOR HUMAN PAPILOMA VIRUS 6 E2 BINDING ASSAY

1. Reagents:

Neutralite Avidin: 20 μl /ml in PBS.

Blocking buffer: 5% BSA, 0.5% Tween 20 in PBS; 1 hr, 25 C.

Assay Buffer: 100 mM KCl, 20 mM HEPES pH 7.6, 0.25 mM EDTA, 1% glycerol, 0.5% NP-40, 50 mM BME, 1 mg/ml BSA, cocktail of protease inhibitors.

^{33}P Full-Length E2 10× stock: 1×10^{-8} "cold" E2 supplemented with 200,000–300,000 cpm of labeled E2, resulting in an approx. specific activity of 200,000–300,000 cpm/1 pmole (M.W. ~100 kD for dimer). Place in microfridge set at 4° C.

Protease inhibitor cocktail (1000×): 10 mg Trypsin Inhibitor (BMB #109894), 10 mg Aprotinin (BMB #236624), 25 mg Benzamidine (Sigma #B-6506), 25 mg Leupeptin (BMB #1017128), 10 mg APMSF (BMB #917575), and 2 mM $NaVo_3$ (Sigma #S-6508) in 10 ml of PBS.

Oligonucleotide stock: (specific biotinylated and sheared salmon sperm (sss)-DNA). Biotinylated oligo at 25 pmole/ml, HPV-E2 1 site TO922/923: (BIOTIN)-CCA GAG TGA CCG AAA ACG GTG TGA GAG C (SEQ ID NO: 68) anti-sense- GGT CTC ACA CCG TIT TCG GTC ACT CTG G (SEQ ID NO: 69) and sss-DNA at 25 μg/ml in assay buffer.

2. Preparation of assay plates:

Coat with 100 μl of stock N-Avidin per well overnight at 4° C.

Wash 2× with 200 μl PBS.

Block with 150 μl of blocking buffer.

Wash 2× with 200 μl PBS.

3. Assay:

Add 40 μl assay buffer/well.

Add 10 μl compound or extract.

Add 10 μl ^{33}P-E2 (20,000–30,000 cpm/0.1 pmoles/well=1×10^{-9}M final concentration).

Shake at 25 C for 15 min.

Incubate additional 45 min. at 25 C.

Add 40 μl oligo mixture (1 pmole of biotinylated specific oligo and 1 μg of sss-DNA)

Incubate 1 hr at 25 C.

Stop the reaction by washing 4× with 200 μl PBS.

Add 150 μl scintillation cocktail.

Count in Topcount.

4. Controls:

a. Non-specific binding (no oligo added)

b. Specific soluble oligo at 80% inhibition

PROTOCOL FOR Nf-kB BINDING ASSAY (p65/p50)

1. Reagents:

Neutralite Avidin: 50 μg/ml in PBS.

Blocking buffer: 5% BSA, 0.5% Tween 20 in PBS; 1 hr, RT.

Assay Buffer: 100 mM KCl, 20 mM HEPES pH 7.9, 0.5 mM EDTA, 1% glycerol, 0.5% NP-40, 1 mg/ml BSA, 50 mM BME, cocktail of protease inhibitors.

^{33}Pp65/p65/p50 10× stock: 1×10^{-8} "cold" p65/p50 (5×10^{-9}M p65+5×10^{-9} M p50 supplemented with 200,000–300,000 cpm of labeled p65, resulting in an approx. specific activity of 200,000–300,000 cpm/1 pmole. Heterodimer formation is promoted by incubating the mixture for 1 hr at 37° C prior to use.

Protease inhibitor cocktail (1000×): 10 mg Trypsin Inhibitor (BMB #109894), 10 mg Aprotinin (BMB #236624), 25 mg Benzamidine (Sigma #B-6506), 25 mg Leupeptin (BMB #1017128), 10 mg APMSF (BMB #917575), and 2 mM $NaVo_3$ (Sigma #S-6508) in 10 ml of PBS.

Biotinylated oligo: 40× stock at 1 pmoles/1 μl in assay buffer. ELAM 2 site END-126/127: (BIOTIN)-CAA CAG ATT GGG GAT ITC CTC GGT TCC ATT GGG GAT TTC CTC CAG C (SEQ ID NO: 70) anti-sense-GC TGA GAG GAA ATC CCC AAT GGA ACC GAG GAA ATC CCC AAT CTG TTG (SEQ ID NO: 71)

2. Preparation of assay plates:

Coat with 100 μl of stock N-Avidin per well overnight at 4° C.

Wash 2× with 200 μl PBS.

Block with 150 μl of blocking buffer.

Wash 2× with 200 μl PBS.

3. Assay:

Add 40 μl assay buffer/well.

Add 10 μl compound or extract.

Add 10 μl ^{33}P-p65/p50 (20,000–30,000 cpm/0.1 pmoles/well=1×10^{-9}M final concentration).

Shake at RT for 15 min.

Incubate additional 45 min. at RT.

Add 40 μl biotinylated oligo (1.0 pmole/40 μl /well) in assay buffer.

Incubate 1 hr at RT.

Stop the reaction by washing 4× with 200 μl PBS.

Add 150 μl scintillation cocktail.

Count in Topcount.

PROTOCOL FOR Nf-kB BINDING ASSAY (p65/p65)

1. Reagents:

Neutralite Avidin: 20 μg/ml in PBS.

Blocking buffer: 5% BSA, 0.5% Tween 20 in PBS; 1 hr, RT.

Assay Buffer: 100 mM KCl, 20 mM HEPES pH 7.9, 0.5 mM EDTA, 1% glycerol, 0.5% NP-40, 1 mg/ml BSA, 50 mM BME, cocktail of protease inhibitors.

^{33}Pp65/p65 10× stock: 1×10^{-8} "cold" p65 supplemented with 200,000–300,000 cpm of labeled p65, resulting in an approx. specific activity of 200,000–300,000 cpm/ 1 pmole.

Protease inhibitor cocktail (1000×): 10 mg Trypsin Inhibitor (BMB #109894), 10 mg Aprotinin (BMB #236624), 25 mg Benzamidine (Sigma #B-6506), 25 mg Leupeptin (BMB #1017128), 10 mg APMSF (BMB #917575), and 2 mM $NaVo_3$ (Sigma #S-6508) in 10 ml of PBS.

Biotinylated oligo: 40× stock at 1 pmoles/1 μl in assay buffer. ELAM 2 site END-126/127: (BIOTIN) -CAA CAG ATE GGG GAT TTC CTC GGT TCC ATE GGG GAT TEC CTC CAG C (SEQ ID NO: 70) anti-sense-GC TGA GAG GAA ATC CCC AAT GGA ACC GAG GAA ATC CCC AAT CTG TTG (SEQ ID NO: 72)

2. Preparation of assay plates:

Coat with 100 μl of stock N-Avidin per well overnight at 4° C.

Wash 2× with 200 μl PBS.

Block with 150 μl of blocking buffer.

Wash 2× with 200 μl PBS.

3. Assay:

Add 40 μl assay buffer/well.

Add 10 μl compound or extract.

Add 10 μl ^{33}P-p65 (20,000–30,000 cpm/0.1 pmoles/well= 1×10^{-9}M final concentration).

Shake at RT for 15 min.

Incubate additional 45 min. at RT.

Add 40 μl biotinylated oligo (1.0 pmole/50 μl /well) in assay buffer.

Incubate 1 hr at RT.

Stop the reaction by washing 4× with 200 μl PBS.

Add 150 μl scintillation cocktail.

Count in Topcount.

PROTOCOL FOR HERPES SIMPLEX VIRUS VP-16 BINDING ASSAY

1. Reagents:

Neutralite Avidin: 20 μg/ml in PBS.

Blocking buffer: 5% BSA, 0.5% Tween 20 in PBS; 1 hr, 25C.

Assay Buffer: 100 mM KCl, 20 mM HEPES pH 7.6, 0.25 mM EDTA, 1% glycerol, 0.5% NP-40, 50 mM BME, 1 mg/ml BSA, cocktail of protease inhibitors.

^{33}P Truncated VP-16/HCF/OCT-1 10× stock mix: 1×10^{-8} "cold" VP-16 supplemented with 250,000–300,000 cpm of labeled VP-16, resulting in an approx. specific activity of 250,000–300,000 cpm/1 pmole (M.W. ~18 kD), 50 μl HCF, and 500 ng of OCT-1 per ml of the stock mix. Place in microfridge set at 4° C.

Protease inhibitor cocktail (1000×): 10 mg Trypsin Inhibitor (BMB #109894), 10 mg Aprotinin (BMB #236624), 25 mg Benzamidine (Sigma #B-6506), 25 mg Leupeptin (BMB #1017128), 10 mg APMSF (BMB #917575), and 2 mM $NaVo_3$ (Sigma #S-6508) in 10 ml of PBS.

Oligonucleotide stock: (specific biotinylated and sss-DNA). Biotinylated oligo at 25 pmole/ml, HSV-VP-16 TO876/877: sen se-Biotin T-GAT AGT CAG GAC TGA ATG CCG TGC ATG CTA ATG ATA TTC TIT GCT TGA TC (SEQ ID NO: 73); anti-sense- GAT CAA GCA AAG AAT ATC ATT AGC ATG CAC GGC ATT CAG TCC TGA CTA TC (SEQ ID NO: 74) and sss-DNA at 2.5 μg/ml in assay buffer.

2. Preparation of assay plates:

Coat with 120 μl of stock N-Avidin per well overnight at 4° C.

Wash 2× with 200 μl PBS.

Block with 150 μl of blocking buffer.

Wash 2× with 200 μl PBS.

3. Assay:

Add 40 μl assay buffer/well.

Add 10 μl compound or extract.

Add 10 μl ^{33}P-VP-16, HCF, OCT-1 stock (25,000–30,000 cpm/0.1 pmoles/well=1×10^{-9}M final concentration, 0.5 μl HCF, and 5 ng OCT-1)

Shake at 25 C for 15 min.

Incubate additional 45 min. at 25 C.

Add 40 μl oligo mixture (1 pmole of biotinylated specific oligo and 100 ng of ss-DNA)—Incubate 1 hr at 25 C.

Stop the reaction by washing 4× with 200 μl PBS.

Add 150 μl scintillation cocktail.

Count in Topcount.

4. Controls for all assays (located on each plate):

a. Non-specific binding (no oligo added)

b. Specific soluble oligo at 80% inhibition.

PROTOCOL FOR HIV TAT BINDING ASSAY

1. Reagents:

Neutralite Avidin: 20 μg/ml in PBS.

Blocking buffer: 5% BSA, 0.5% Tween 20 in PBS; 1 hr, RT.

Assay Buffer: 100 mM KCl, 20 mM HEPES pH 7.9, 0.5 mM EDTA, 1% glycerol, 0.5% NP-40, 1 mg/ml BSA, 50 mM BME, cocktail of protease inhibitors.

^{33}PTAT10× stock: 1×10^{-8} "cold" p65 supplemented with 200,000–300,000 cpm of labeled TAT, resulting in an approx. specific activity of 200,000–300,000 cpm/1 pmole.

Protease inhibitor cocktail (1000×): 10 mg Trypsin Inhibitor (BMB #109894), 10 mg Aprotinin (BMB #236624), 25 mg Benzamidine (Sigma #B-6506), 25 mg Leupeptin (BMB #1017128), 10 mg APMSF (BMB #917575), and 2 mM $NaVo_3$ (Sigma #S-6508) in 10 ml of PBS.

Biotinylated oligo: 40× stock at 1 pmoles/1 μl in assay buffer. TAR RNA site: (BIOTIN) -GGG TCT CTC TGG TTA GAC CAG ATC TGA GCC TGG GAG CTC TCT GGC TAA CTA GGG AAC CCA (SEQ ID NO: 75)

2. Preparation of assay plates:

Coat with 100 μl of stock N-Avidin per well overnight at 4° C.

Wash 2× with 200 μl PBS.

Block with 150 μl of blocking buffer.

Wash 2× with 200 μl PBS.

3. Assay:

Add 40 μl assay buffer/well.

Add 10 μl compound or extract.

Add 10 μl ^{33}P-pTAT (20,000–30,000 cpm/0.1 pmoles/well= 1×10^{-9}M final concentration).

Shake at RT for 15 min.

Incubate additional 45 min. at RT.

Add 40 μl biotinylated oligo (1.0 pmole/50 μl /well) in assay buffer.

Incubate 1 hr at RT.

Stop the reaction by washing 4× with 200 μl PBS.

Add 150 μl scintillation cocktail.

Count in Topcount.

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference. Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the an in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i i i) NUMBER OF SEQUENCES: 75

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TTTCATATTA CTCT 14

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TGCGTGAGAA GA 12

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:3:

YCAGCTGYGG 10

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:4:

AGAACANNNT GTTCT 15

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TGANCCCTTG ACCCCT 16

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GGGGGGGGGG GGGGGG 16

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GACGCANYGR WNNNMG 16

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:8:

ACACCCAAAT ATGGCGAC 18

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GTGTCAAAGG TCA 13

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:10:

YNNNNNNRRC CAATCANYK 19

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:11:

YAGYNNNRRC CAATCNNNR 19

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CCGCCCCCGC 10

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:13:

AGGTCANNNT GACCT 15

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GTGTCAAAGG TCA 13

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CAGCCCCCGC GCAGC 15

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:16:

AGAACANNNT GTTCT 15

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GCACCAATCA CAGCGCGC 18

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:18:

TCAGGTCACA GTGACCTGA 19

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:19:

TGGGGATTCC CCA 13

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:20:

GTTAATNATT AAC 13

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:21:

TATTGAYTTW G 11

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:22:

KGCWARGKYC AY 12

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:23:

NGAANNGAAN NGAAN 15

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:24:

GCCATCTGCT 10

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:25:

CGGGAAATGG AAACTG 16

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:26:

CTTTCAGTTT 10

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:27:

CTTTCTCTTT 10

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:28:

GCTTCAGTTT 10

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:29:

TGGGGATTCC CCA 13

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:30:

GCCTGCAGGC 10

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:31:

GTTAATNATT AAC 13

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:32:

GCGCCCTTTG GACCT 15

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:33:

YTAAAAATAA YYY 13

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:34:

TGGGGATTCC CCA 13

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:35:

YTAWAAATAR 10

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:36:

AGAACANNNT GTTCT 15

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(i x) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..14
(D) OTHER INFORMATION: /note="Where this sequence can contain 5 or 6 N nucleotides"

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:37:

YGGMNNNNNG CCAA 14

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:38:

GGAGGAAAAA CTGTTTCAT 19

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:39:

GRGRTTKCAY 10

(2) INFORMATION FOR SEQ ID NO:40:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:40:

GGGAMTNYCC 10

(2) INFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:41:

AGGTCATGAC CT 12

(2) INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:42:

CACCGTTCCG CTCTAGATAT CTC 23

(2) INFORMATION FOR SEQ ID NO:43:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:43:

AGAAAGGGAA AGGA 14

(2) INFORMATION FOR SEQ ID NO:44:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:44:

AGAACANNNT GTTCT 15

(2) INFORMATION FOR SEQ ID NO:45:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:45:

AAGTGAAAGT 10

(2) INFORMATION FOR SEQ ID NO:46:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(i x) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..18
(D) OTHER INFORMATION: /note="Where N is one or more nucleotides."

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:46:

ATGGGANCTC AGCTGTGC 18

(2) INFORMATION FOR SEQ ID NO:47:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:47:

AGGTCATGAC CT 12

(2) INFORMATION FOR SEQ ID NO:48:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:48:

CCCCTAGCAA CAGATG 16

(2) INFORMATION FOR SEQ ID NO:49:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:49:

AAGATAAAAC C 11

(2) INFORMATION FOR SEQ ID NO:50:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:50:

GGATGTCCAT ATTAGGACAT CT 22

(2) INFORMATION FOR SEQ ID NO:51:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:51:

AAGTGTTTGC 10

(2) INFORMATION FOR SEQ ID NO:52:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:52:

AGGAAGTTCC 10

(2) INFORMATION FOR SEQ ID NO:53:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:53:

TCTTCTCACG CAACT 15

(2) INFORMATION FOR SEQ ID NO:54:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:54:

CACCTGNNNN TTTCCC 16

(2) INFORMATION FOR SEQ ID NO:55:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:55:

ATTTTTCTGA TTGGCCAAAG 20

(2) INFORMATION FOR SEQ ID NO:56:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:56:

GGTTAGCATA TGCTAACCA 19

(2) INFORMATION FOR SEQ ID NO:57:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:57:

ACCGAAAACG GTGT 14

(2) INFORMATION FOR SEQ ID NO:58:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:58:

ATGCTAATGA TA 12

(2) INFORMATION FOR SEQ ID NO:59:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:59:

GGGTCTCTCT GGTTAGACCA GATCTGAGCC TGGGAGCTCT CTGGCTAACT AGGGAACCCA 60

(2) INFORMATION FOR SEQ ID NO:60:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:60:

GGATCTGGTT AGCATATGCT AACCAGGATC 30

(2) INFORMATION FOR SEQ ID NO:61:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:61:

GGATCTGGTA CCGAAAACGG TACCAGGATC 30

(2) INFORMATION FOR SEQ ID NO:62:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:62:

GGATCTGGTT AGTTAGCAAT GACCAGGATC 30

(2) INFORMATION FOR SEQ ID NO:63:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:63:

GGATCTGGTT AGGGGATTTC CACCAGGATC 30

(2) INFORMATION FOR SEQ ID NO:64:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:64:

GGATCTGGTT ATGCTAATGA TATCAGGATC 30

(2) INFORMATION FOR SEQ ID NO:65:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:65:

GATCTTGGTT AGCATATGCT AACCAGATCC 30

(2) INFORMATION FOR SEQ ID NO:66:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:66:

TATCTACATT AGCAATGCCT TAGCAATGTG CATA 34

(2) INFORMATION FOR SEQ ID NO:67:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:67:

TATGCACATT GCTAAGGCAT TGCTAATGTA GATA 34

(2) INFORMATION FOR SEQ ID NO:68:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:68:

CCAGAGTGAC CGAAAACGGT GTGAGACC 28

(2) INFORMATION FOR SEQ ID NO:69:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:69:

GGTCTCACAC CGTTTTCGGT CACTCTGG 28

(2) INFORMATION FOR SEQ ID NO:70:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 46 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:70:

CAACAGATTG GGGATTTCCT CGGTTCCATT GGGGATTTCC TCCAGC 46

(2) INFORMATION FOR SEQ ID NO:71:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 47 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:71:

GCTGAGAGGA AATCCCCAAT GGAACCGAGG AAATCCCCAA TCTGTTG 47

(2) INFORMATION FOR SEQ ID NO:72:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 47 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:72:

GCTGAGAGGA AATCCCCAAT GGAACCGAGG AAATCCCCAA TCTGTTG 47

(2) INFORMATION FOR SEQ ID NO:73:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:73:

GATAGTCAGG ACTGAATGCC GTGCATGCTA ATGATATTCT TTGCTTGATC 50

(2) INFORMATION FOR SEQ ID NO:74:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:74:

GATCAAGCAA AGAATATCAT TAGCATGCAC GGCATTCAGT CCTGACTATC 50

(2) INFORMATION FOR SEQ ID NO:75:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:75:

GGGTCTCTCT GGTTAGACCA GATCTGAGCC TGGGAGCTCT CTGGCTAACT AGGGAACCCA 60

What is claimed is:

1. A method of screening for a compound which inhibits the binding of a transcription factor to a nucleic acid, said method comprising the steps of:

forming a mixture by combining a labeled protein comprising a portion of a transcription factor and a label, a nucleic acid conjugate, said compound and a receptor immobilized on a solid substrate, wherein said nucleic acid conjugate comprises a nucleotide sequence and a ligand which specifically binds said receptor;

incubating said mixture under conditions whereby said receptor is bound to said ligand, and wherein in the absence of said compound said labeled protein is sequence-specifically bound to said nucleic acid conjugate;

separating from said solid substrate a fraction of said mixture, which fraction comprises said labeled protein if said labeled protein is not sequence-specifically bound to said nucleic acid conjugate; and

detecting the presence or absence of said label on said solid substrate,

wherein the absence of said label on said solid substrate indicates said compound inhibits the binding of said transcription factor to said nucleic acid.

2. A method according to claim 1, wherein said labeled protein sequence-specifically binds said nucleic acid conjugate with a binding affinity of at least 10^6 M^{-1}.

3. A method according to claim 1, wherein said mixture further comprises a portion of an auxiliary protein which enhances binding of said labeled protein to said nucleic acid conjugate.

4. A method according to claim 1, wherein said transcription factor is a viral transcription factor.

5. A method according to claim 1, wherein said transcription factor is a bacterial transcription factor.

6. A method according to claim 1, wherein said transcription factor is a plant transcription factor.

7. A method recording to claim 1, wherein transcription factor is a fungal transcription factor.

8. A method according to claim 1, wherein said transcription factor is a monocotyledonous or dicotyledonous plant transcription factor.

9. A method according to claim 1, wherein said receptor is avidin and said ligand is biotin.

10. A method according to claim 1, wherein said label is a radioactive phosphorous atom.

11. A method according to claim 1, wherein said forming step and said separating step are performed at least in part by a computer controlled electromechanical robot.

12. A method according to claim 11, wherein said robot comprises an axial rotatable arm and said solid substrate is a microliter plate.

13. A kit for screening for a compound which inhibits the binding of a transcription factor to a nucleic acid, said kit comprising:

a solid substrate,

a labeled protein comprising a portion of a transcription factor and a label,

said compound,

a receptor immobilized on said solid substrate, and

a nucleic acid conjugate comprising a nucleotide sequence and a ligand which specifically binds said receptor.

14. A kit according to claim 13, further comprising a computer-controllable electromagnetic robot with an axial rotatable arm and wherein said solid substrate is a microtiter plate.

* * * * *